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(54) Title: CO–EXPRESSION OF G_{ia} PROTEIN AND G_i PROTEIN COUPLED RECEPTOR TO ENHANCE SIGNAL TRANSDUCTION RESPONSES

(57) Abstract

A method for detecting compounds that bind to a G_i protein coupled receptor which comprises the steps of culturing a cell in a medium under conditions wherein G_{ia} protein and a G_i protein coupled receptor are co-expressed, contacting the cell with a test compound, and assaying the cell for a cellular response to the compound, the response being an increase in the cytoplasmic calcium concentration, where the cell has been transfected with a gene encoding aG_i protein coupled receptor and a gene coding for G_{ia} protein capable of coupling to said G_i protein coupled receptor. Cells useful in such methods, and methods for making such cells, are also provided.

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CO-EXPRESSION OF G_{ia} PROTEIN AND G_i PROTEIN COUPLED RECEPTOR TO ENHANCE SIGNAL TRANSDUCTION RESPONSES

FIELD OF THE INVENTION

The invention relates to methods for rapidly assaying for agonism or antagonism of G_i protein coupled receptors and to cells which can serve as assay systems for evaluating such agonism or antagonism. The invention relates particularly to methods for rapidly assaying for agonist and antagonist compounds which bind to G_i protein coupled receptors which stimulate intracellular calcium mobilization, such as the nociceptin receptor, and particularly chemokine receptors such as CCR3, CCR2, and the interleukin 8 receptor type B.

BACKGROUND OF THE INVENTION

G proteins (guanine nucleotide binding regulatory proteins) are integral parts of regulatory mechanisms that operate in all human cells. Impairing G protein function can affect a cell's response to hormonal signals, e.g., by interfering with intracellular metabolic pathways. This can cause development or maintenance of a wide variety of disease states.

When functioning normally, G proteins act as essential parts of signal transducing mechanisms by which extracellular hormones and neurotransmitters convey their signals through the plasma membrane of the cell and thus elicit appropriate intracellular responses.

These signal transducing mechanisms comprises three distinct components:

1) a receptor protein with an extracellular binding site specific for a given agonist or hormone;

2) a membrane-bound effector protein that when activated catalyzes the formation or facilitates the transport of an intracellular second messenger, such as adenylyl cyclase which converts ATP to cyclic AMP (cAMP); and

3) a protein which functions as a communicator between these two.

G proteins fulfill this function as communicator between receptor and effector proteins in the generation of intracellular responses to extracellular hormones and agonists.

G proteins are composed of three polypeptide subunits: G alpha (or G_a), G beta (or G_b) and G gamma (or G_g). The G_b and G_g polypeptide subunits occur in living cells as a heterodimer, commonly referred to as a "bg" dimer. The conformation of these subunits and their degree of association with each other change during the signal transducing mechanism. These changes are associated with the hydrolysis of the nucleotide guanosine triphosphate (GTP) to form guanosine diphosphate (GDP) and free phosphate (GTPase activity). The binding sites for GTP and GDP, and the GTPase catalytic site, reside in the alpha subunit of the G protein.

An example of a G protein cycle which occurs when a signal is conveyed across the cell membrane is as follows:

In an unstimulated cell the G proteins are found in a resting state in which alpha, beta and gamma subunits are complexed together and GDP is bound to G_a. The binding of an appropriate hormone or agonist to the receptor changes its conformation and causes it to activate the G protein by displacing GDP and allowing GTP to bind. This is the rate-limiting step in the G protein cycle. When GTP is bound to G_a it may dissociate from bg and is able to bind to, and activate, adenylate cyclase, which releases cAMP into the cytoplasm. GTP is then hydrolysed to GDP G_a and the cycle is complete.

A series of complex interactions has evolved to allow signal amplification, such that a single hormone-receptor complex can trigger the production of several hundred second messenger molecules, such as cAMP. cAMP is a potent second messenger that binds to and activates protein kinase A (PKA). PKA was first shown to play a role in glycogen metabolism and is now known to influence a variety of processes, including transcription.

This system also allows several different receptors to interact with a signal-generating enzyme. The receptors can activate the enzyme or inhibit it. For example, distinct alpha subunits G_{sa} (stimulatory) and G_{ia} (inhibitory) combine with the bg complex to form stimulatory or inhibitory G proteins. An example of a receptor that interacts with G_i to lower the concentration of cAMP is the alpha 2-adrenergic receptor. Integration of signals from G_s and G_i allows the level of cAMP in the cell to be fine-tuned in response to several different extracellular agonists.

Although G proteins were first identified and characterized in relation to the adenylyl cyclase system discussed above, it is now known that they are involved in many aspects of cell signaling. In particular, certain G proteins act in signal transducing pathways that activate phospholipase C. This enzyme catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3). DG activates protein kinase C (PKC) which phosphorylates a certain sub-set of cellular proteins and modulates their activity. For example, PKC is important in controlling intracellular pH and in transcriptional activation of specific genes. IP_3 is a small water-soluble molecule that causes the release of calcium ions (Ca^{2+}) from intracellular stores. Ca^{2+} is a potent intracellular messenger in several metabolic and homeostatic pathways.

Many drugs are currently directed at hormone receptors. Examples include beta-adrenergic agents used in the treatment of asthma, and "beta-blockers" used in the treatment of high blood pressure. Assays for chemical compounds that act at particular G protein coupled receptors have typically been based on the use of radioligands, where either the drug of interest is radiolabeled and its binding to receptor directly assayed, or the ability of a compound of interest to displace a known radioligand from a receptor of interest is assayed. Radioligand binding assays, however, use dangerous and expensive radioisotopes, and require disposal of radioactive reagents and supplies. Also, these assays are not as amenable to high-throughput systems as are assays which monitor intracellular processes such as calcium release.

Various methods have been developed to assay for receptor/ligand interactions without using radioactive reagents. For example, to assay G protein coupled signal transduction pathways that involve the release of intracellular calcium stores, direct detection methods have been developed that precipitate calcium with alizarin sulfonate.

Receptors are normally present in very low numbers on the surfaces of cells that are useful in such assays. Therefore, when performing receptor activation assays it is common to first overexpress the receptor of interest by introducing into cells DNA which is capable of expressing the receptor.

However, merely overexpressing the desired receptor may not allow generation of sufficient signal. For example, when the receptor is activated, it may

not transduce sufficient intracellular calcium to allow unambiguous detection. This problem has been found by the present inventors to be particularly present with respect to detection of intracellular calcium release associated with the activation of G_i coupled receptors.

Thus, when G_i coupled receptors are overexpressed in cells, the calcium release achieved by exposure of the cells to receptor agonist or antagonist compounds can be too low for reliable measurement, i.e., the resulting signal strength may be too low for an efficient assay. Thus, there is a need for a method to increase the second messenger signal strength generated by agonist binding to receptor-overexpressing cells. The signal generated by agonist binding must be sufficiently robust that a decrease in signal, e.g., in response to antagonist binding, is detectable.

SUMMARY OF THE INVENTION

It has been determined that expression of transfected DNA encoding a G_{ia} protein subunit that couples to a co-expressed receptor that couples to a G_i protein will obviate the "signal strength" problem referred to above, and will produce a significant and reproducible intracellular calcium signal that can be reliably detected.

In one aspect, the invention relates to a method for assaying a test compound for its effect on a G_i protein coupled receptor. The method involves contacting a cell with the test compound and assaying the cell for cytoplasmic calcium concentration. According to the invention, the cell employed in such an assay has been transfected with a gene encoding a G_i protein coupled receptor and with a gene coding for a G_{ia} protein capable of coupling to the G_i protein coupled receptor to increase cytoplasmic calcium upon binding of an agonist to the G_i protein coupled receptor.

In another aspect, the present invention relates to a cell useful for high- or low-throughput assays to determine if a compound binds to a G_i protein coupled receptor, the cell comprising transfected DNA which expresses a G_i coupled receptor, transfected DNA which expresses a G_{ia} protein subunit, and an intracellular calcium release mechanism which produces a detectable signal as measurable by fluorescence of a fluorophore in response to agonist compound binding to the receptor when the G_i protein coupled receptor is coexpressed with the G_{ia} protein subunit.

In yet another aspect, the invention relates to a method for producing a cell for detecting compounds which bind to a G_i protein coupled receptor which

comprises the steps of transfecting a cell with a gene which codes for a G_i protein coupled receptor, transfecting the cell with a gene which codes for a G_{ia} protein capable of coupling to the receptor, and culturing the cell in medium under conditions in which the G_{ia} protein and the G_i protein coupled receptor are co-expressed in the cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is a depiction of the pHEBo vector.

Figure 1b is a depiction of the p394 vector.

Figure 1c is a depiction of the pcmvmcs1 vector.

Figure 1d is a depiction of the pcDM8 vector.

Figure 1e is a depiction of the pm3ar vector.

Figure 1f is a depiction of the pm3CCR3 vector.

Figure 1g is a schematic showing the modification of the CCR3 gene by the addition of a signal sequence coding region.

Figure 1h is a depiction of the pm3CCR3sp vector.

Figure 2a is a depiction of the pE3 vector.

Figure 2b is a depiction of the pE3delta vector.

Figure 2c is a depiction of the pPur vector.

Figure 2d is a depiction of the pE3pur vector.

Figure 2e is a depiction of the pEpurGia2 vector.

Figure 3 is the nucleotide sequence of the pCEP4 vector.

Figure 4 is the nucleotide sequence of the pCDM8 vector.

Figure 5 is the nucleotide sequence of the pBSIISK+ vector.

Figure 6 is the nucleotide sequence of the pPur vector.

Figure 7 is a bar graph illustrating the stability of the Kd for agonist in CCR3 receptor and CCR3 receptor/G_{ia2} protein transfected cells.

Figure 8 is a bar graph illustrating the stability of the receptor number per cell in CCR3 receptor and CCR3 receptor/G_{ia2} protein transfected cells.

Figure 9 is a bar graph illustrating the increases in calcium mobilization in response to eotaxin either in cells transfected with the CCR3 receptor alone or in cells cotransfected with the CCR3 receptor and G_{ia2} protein.

Figure 10 is a bar graph illustrating the increases in calcium mobilization in response to nociceptin either in cells transfected with the nociceptin

receptor alone or in cells cotransfected with the nociceptin receptor and G_{ia2} protein.

Figure 11 is a graph representing a time course illustrating the increase in the concentration of intracellular calcium in response to the CCR2 agonist MCP-1 either in cells transfected with the CCR2 receptor alone or in cells cotransfected with the CCR2 receptor and G_{ia2} protein.

Figure 12 is graph showing the dose-response relationship for increases in cytoplasmic calcium as a result of exposure of cells to interleukin 8 either in cells transfected with the interleukin 8 receptor type B alone or in cells cotransfected with the interleukin receptor type B and G_{ia2} protein.

DETAILED DESCRIPTION OF THE INVENTION

All patents, patent applications, and publications referred to herein are hereby incorporated by reference in their entirety. In case of a conflict in description or terminology, the present disclosure is intended to control.

Definitions:

"G_i protein" as used herein refers to the heterotrimeric guanine nucleotide binding proteins G_{i1}, G_{i2}, and G_{i3}, including those from any animal species (e.g. mouse), and derivatives having mutated sequences such as G_{ip2}.

"Transfecting" as used herein means to introduce exogenous DNA into a cell.

"Codes for" as used herein means the property of a DNA polynucleotide to be transcribed by an RNA polymerase into messenger RNA, which messenger RNA can in turn be translated into a polypeptide. The polypeptide so produced is said to be "coded for" by the DNA polynucleotide.

G protein "coupling" to a receptor as used herein means the activation of a guanine nucleotide binding protein by the interaction of a cell surface receptor with an agonist for that receptor.

"Culturing" as used herein means the incubation of cells in a medium sufficient for the maintenance of cell division and cellular physiological processes.

"Co-expressed" as used herein means that at least two proteins are synthesized in a cell as a result of the introduction into the cell of DNA foreign to the cell.

"Cellular response" as used herein means the response of a cell to the activation of a G protein by an agonist-bound receptor, or the response of a cell to the

presence of a receptor antagonist, and includes second messenger responses such as the opening or closing of ionic channels, such as calcium or potassium channels, the activation or inhibition of adenylyl cyclase, which catalyzes the conversion of ATP to cyclic AMP, and the activation of phospholipase C, which cleaves phosphatidylinositol 4,5 bisphosphate into diacylglycerol and inositol triphosphate (IP_3).

"Native DNA" of a cell or cell line is the DNA complement of a defined cell or cell line prior to the introduction, by any means, of exogenous DNA into the cell or cell line.

"Integrated" as used herein with regard to DNA refers to DNA that has been transfected into a cell which has been incorporated into the native DNA of a cell.

"Stably transfected" as used herein means the introduction into and maintenance of exogenous DNA into a cell or cell line by integration into the host chromosome or by episomal transfection for at least 2 weeks and preferably more than 4 months.

"Transiently transfected" as used herein means the introduction into and maintenance of exogenous DNA in a cell or cell line for a limited period of time. If the DNA is derived from an integrating construct, then transience is defined as 0 to 5 days post transfection or until detectable signal has faded into insignificance. If the DNA is derived from a replicating, episomal construct, then transience is defined as 0 to 21 days post transfection, or until detectable signal has faded into insignificance. A detectable signal can be the direct detection of the transfected DNA, for example by Southern blotting or PCR, or detection of an RNA transcribed from the DNA, or detection of a protein expressed by the DNA.

A "DNA construct" as used herein means a polynucleotide which contains an open reading frame which encodes a protein, and includes any promoter, transcription initiation, transcription termination, or other nucleotide sequences which might facilitate expression of the encoded protein.

An "intracellular calcium release mechanism" as used herein means any combination of cellular proteins which, when activated, function to cause the release of calcium ions from intracellular stores into the cytoplasm of the cell.

G_i protein coupled receptors, such as the C-C chemokine receptor 3 (CCR3), C-C chemokine receptor 2 (CCR2), interleukin 8 receptor type B (CXCR2), and nociceptin receptor (NociR), must physically couple to G_i proteins in order to

transduce extracellular stimuli into intracellular signals that lead to functional responses. We have developed an assay for detecting agonists and antagonists of G_i protein coupled receptors. The assay is particularly useful in high throughput screening to determine compounds that are useful as drug candidates.

When expressed from exogenously introduced DNA constructs, G_i protein coupled receptors can be expressed at low levels on the surface of the transfected cells; they may also overwhelm the endogenous cellular mechanism for transducing extracellular signals into intracellular responses. The signals generated by activation of such expressed receptors can be weak. We have determined that when performing assays relying on mobilization of calcium from intracellular stores, weakness in signal strength can be obviated by transfecting cells which express G_i protein coupled receptors with a DNA construct which encodes a G_{ia} protein subunit capable of coupling to the receptor. This can be done, for example, by cotransfecting cells with separate constructs encoding the G_i protein coupled receptor and G_{ia} protein subunit, or by transfecting a single construct which encodes both a G_i protein coupled receptor and a G_{ia} protein subunit.

Coexpression of the G_{ia} subunit has been determined to have a positive effect on intracellular calcium mobilization. It has been determined in particular that G_{ia} protein subunits transduce signal generated by G_i coupled receptors to release intracellular calcium stores. It is known that G protein bg dimers are required in the signal transduction pathway from G_i coupled receptors to the activation of phospholipase C (which ultimately results in the release of intracellular calcium stores). See, e.g., Wu, D. et al., *Science* 261:101 (1993); Slepak, V.Z. et al., *J. Biol. Chem.* 270:4037 (1995); and Arai, H. et al., *Proc. Natl. Acad. Sci.* 94:14495 (1997). We have determined that expression of G_{ia} protein subunits can act to stimulate calcium release in response to receptor activation. Furthermore, expression of bg dimers or phospholipase C b (PLCb) is not required.

DNA, VECTORS, and HOST CELLS

In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA are used. Such techniques are well known and are explained fully in, for example, Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); *Nucleic Acid Hybridization*, 1985, (Hames and Higgins); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *Animal Cell Culture*, 1986 (R.I. Freshney ed.); *Immobilized Cells and Enzymes*, 1986 (IRL Press); Perbal, 1984, *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic Press, Inc.); *Gene Transfer Vectors for Mammalian Cells*, 1987 (J. H. Miller and M. P. Calos eds., Cold Spring Harbor Laboratory); and *Methods in Enzymology* Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively).

Insertion of nucleic acids (typically DNAs) encoding the receptor and G_{ia} polypeptides into a vector to create a DNA construct for transfection is easily accomplished when the termini of both the DNAs and the vector comprise compatible restriction sites. If this cannot be done, it may be necessary to modify the termini of the DNAs and/or vector by digesting back single-stranded DNA overhangs generated by restriction endonuclease cleavage to produce blunt ends, or to achieve the same result by filling in the single-stranded termini with an appropriate DNA polymerase.

Alternatively, any restriction site desired may be produced, e.g., by ligating nucleotide sequences (linkers) onto the termini. Such linkers may comprise specific oligonucleotide sequences that define desired restriction sites. Restriction sites can also be generated by the use of the polymerase chain reaction (PCR). See, e.g., Saiki *et al.*, 1988, *Science* **239**:48. The cleaved vector and the DNA fragments may also be modified if required by homopolymeric tailing.

Nucleic acids utilized in the invention may be isolated directly from well-known cells. Alternatively, the polymerase chain reaction (PCR) method can be used to produce the nucleic acids of the invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression. A clone containing DNA expressing G_{ia2} is available from the American Type Culture Collection, Rockville, MD, Cat. No. 63311, and the sequence of G_{ia2} protein is available from Genbank, accession number M13963. The sequence of a G_{ia1} protein is available from Genbank, accession number M17219, and the sequence of a G_{ia3} protein is also available from Genbank, accession

number M20597. A clone containing DNA expressing G_{ip2} is available from the American Type Culture Collection, Rockville, MD, Cat. No. 63312; the DNA has the sequence G_{ia2}Q205L.

The nucleic acids employed in the present invention may be flanked by native regulatory sequences, i.e., those that are found associated with the relevant gene in the genome of the organism from which the gene is isolated, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.).

The invention employs nucleic acid vectors, or DNA constructs comprising nucleic acid sequences which code for the G_{ia} protein or G_i coupled receptors. It is understood that if desired, functional derivatives or fragments thereof may be employed to the same effect.

A large number of well-known vectors can be used in the invention for expression in a variety of eukaryotic hosts. For example, the G_{ia} protein or G_i coupled receptors may be expressed by transfecting known mammalian expression vectors, such as vectors available commercially from, e.g., Invitrogen Corporation, Carlsbad, CA, such as pcDNA1.1 for transient transfection, and pcDNA3.1, pcDNA3.1/zeo or pcDNA3.1/hyg, pRc/RSV or pRc/CMV2 for stable transfection, employing appropriate eukaryotic host cells, and using methods disclosed herein or otherwise known to those skilled in this art.

Recombinant cloning vectors employed in the invention can include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes. The inserted G_{ia} protein and G_i coupled receptor coding sequences may be synthesized by standard methods, isolated from natural sources, or prepared as hybrids. Ligation of the G_{ia} protein and G_i coupled receptor coding sequences to transcriptional regulatory elements and/or to other amino acid coding sequences is achieved by known

methods. Suitable host cells may be transformed/transfected/infected as appropriate by any suitable method including electroporation, $CaCl_2$ mediated DNA uptake, microinjection, and microprojectile based transfection.

A large number of transcription initiation and termination regulatory regions that can be employed in the invention have been isolated by those skilled in this art and shown to be effective in the transcription and translation of heterologous proteins in eukaryotic host cells. These regions, methods of isolating them and ways to manipulate them are well-known in the art.

Advantageously, vectors and DNA constructs employed in the invention also include a transcription regulatory element (i.e., a promoter) operably linked to the nucleotide sequence encoding the protein to be expressed. The promoter may optionally contain operator portions and/or ribosome binding sites. Suitable promoters for mammalian cells include without limitation viral promoters such as those from Epstein-Barr Virus (EBV), Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV), and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly(A) addition sequences. Enhancer sequences, which increase expression, may also be included. Sequences which cause amplification of the gene may also be desirable. These sequences are well described in the art.

Nucleic acids encoding G_{ia} or G_i coupled receptor polypeptides may also be introduced into cells by known recombination methods. For example, such nucleic acids can be introduced into a cell to effect homologous recombination at the site of a corresponding endogenous gene or a sequence with substantial identity to the gene. Other known recombination-based methods such as nonhomologous recombination or deletion of endogenous genes by homologous recombination may also be used.

Preferably, separate episomal plasmids are used for expression of the G_i coupled receptor and the G_{ia} protein subunit. Each such episome preferably contains its own selectable marker. The two episomes may be co-transfected simultaneously into the same cell to produce a pool of stably transformed cells available for use in high-throughput screening assays. Such cells are advantageously obtained within about 2 weeks.

Transformation methods in which either the gene encoding the G_i coupled receptor or the gene encoding the G_{ia} subunit (or both) is integrated into the host nuclear material are less preferred, as the time required to obtain a cell line in this

manner which stably expresses either protein is normally from three to six months. Using the preferred episomal expression system of the invention, the need for clonal selection of stably transfected cells is eliminated, assayable cell lines are generated in weeks rather than months, and the cellular response achievable in response to receptor activation is significantly enhanced.

The preferred episomal expression system of the invention employs episomes bearing an EBV origin of replication in a permissive cell type expressing the EBNA1 protein for recombinant receptor binding assays. Primate and canine cell lines are preferred as host cells. Rodent cells are not preferred since they are not permissive for EBNA1/EBV oriP interactions, i.e., rodent cells do not permit replication of EBV oriP based vectors.

HEK-293 cells are particularly preferred and are available from the American Type Culture Collection (ATCC), Rockville, MD, Accession Number CRL-1573. HEK-293 cells that constitutively express EBNA1 are most preferred, and are commercially available from Invitrogen Corporation, Carlsbad, CA.

If desired, episomes can be used for the construction of integratively stable cell lines that express EBNA1. That is, the episomes of the invention can be used to transfect cells, and rather than replicate independently, the genes encoded on the episomes can be integrated into the chromosomes of the host cell, thereby allowing replication along with the host cell's DNA. If episomes are employed which also express the EBNA1 protein, then any permissive cell line can be used to practice the invention, regardless of whether or not it pre-expresses EBNA1.

The preferred episomally co-transfected cell lines of the invention remain stably transfected for at least 5 months after transfection, preferably as demonstrated by ligand-induced cellular responses, northern blot analysis of steady state RNA levels (encoding receptor and G protein), and western blot analysis of G protein levels.

In order to detect the release of intracellular calcium stores in response to stimulation of G_i coupled receptors by agonists, or the inhibition of such release due to binding of antagonists to G_i coupled receptors, both direct and indirect measurements can be used. Such methods are well-known in this art. For example, direct detection methods are well-known that precipitate calcium with alizarin sulfonate. Other well-known direct detection methods use photoproteins that fluoresce

when bound to calcium, such as aequeorin and obelion. Recently, non-protein fluorescent indicators of calcium release have been developed for use in direct detection; these include Fura-2/AM, Indo-1/AM, and Fluo-3/AM and their derivatives. Such fluorescent probes are commercially available, and can be obtained from Molecular Probes, Eugene, OR; Teflabs, Texas; and Sigma Chemical Co., St. Louis, MO.

Other known methods for direct detection of calcium use metallochromic indicators such as murexide, arsenazo III, and antipyrylazo III.

Calcium release can be indirectly measured using patch clamps (which record calcium release in single cells), mini/microelectrodes, and vibrating calcium electrodes.

Confocal microscopy permits sensitive visual detection of fluorescence due to calcium. Methods of screening using confocal microscopy are described in U.S. patent application Serial No. 08/868,280 filed June 3, 1997.

The method of the invention can be carried out as part of a high throughput screening of a library of compounds for binding to receptor. In one embodiment of the invention, the method is carried out with a plurality of compounds to be screened, preferably at least about 96 compounds, such as when using a 96 well microtitre plate. Such assays can also be performed in the 1536 well plate described in U.S. patent application serial no. 60/037,636, filed February 18, 1997. The library of compounds to be screened can be quite large, e.g., containing more than 100,000 compounds.

It is preferred that the compounds assayed in the high throughput method be derived from combinatorial libraries on polymer beads. By synthesizing sufficient compound on each bead for a few assays, compound handling is reduced or eliminated.

Preferably, the library compounds are eluted from the beads and evaporated to dryness in microtiter plates in preparation for the assay. Compounds on beads can be released by photocleavage, or another type of cleavage. Cleavage of photocleavable linkers is preferred. Such linkers, and methods for their cleavage, are described in Barany et al. (1985) *J. Am. Chem. Soc.* 107:4936. Examples of other linkers and the relevant cleavage reagents are described in WO 94/08051.

Using combinatorial libraries prepared on beads, the identity of active

compounds is preferably determined using the encoding system described in WO 94/08051 and in WO 95/30642. In this system, chemical tags encoding the identities of the compounds are applied to the solid supports. The identity of the compound on a given support can be determined by detaching the chemical tags from the support, identifying the tags by, e.g., gas chromatography, and correlating the identities of tags with the identity of the compound. Once an active compound is identified, the corresponding bead (which had contained the compound) can be examined, and the identity of the compound determined by releasing the tags and decoding by this method.

The following examples are intended to illustrate the invention only, and are not intended to be limiting in any way. Many variations and adaptations of the present invention will be apparent to those of ordinary skill in the art, and these are intended to be included within the scope of the invention.

EXAMPLE 1: CONSTRUCTION OF EPISOMAL EXPRESSION VECTORS

A. Construction of pHEBO Vector

The pHEBo vector was made using commercially available vectors. The sequence of vector pBR322 (Genbank accession number synpbr322) from nucleotide 1 to nucleotide 772 was ligated to the nucleotide sequence of vector pCEP4, SEQ ID NO: 1, Figure 3, from position 8146 to 10376 (Invitrogen, Carlsbad, CA, Cat. No. V004-50). To this construct was ligated pCEP4 nucleotides 1333 to 5500. Prior to ligation, fragments were PCR amplified or joined using preexisting restriction sites. The resulting plasmid contained the Epstein Barr Virus (EBV) origin of replication (oriP), a hygromycin resistance marker (hyg) transcribed from the minimal Herpes Simplex Virus (HSV) thymidine kinase (tk) promoter, and was terminated with the tk poly adenylation signal (poly(A)), in vector pBR322. The pHEBo vector is shown schematically in Figure 1a.

B. Construction of pcmvmcs1 Vector

Vector p394 was constructed according to Colberg-Poley, A.M. et al. *J Virol.* 1992 Jan; 66(1): 95-105. Briefly, the vector can be made by cloning the CMV IE promoter (which can be obtained from vector pCEP4, SEQ ID NO: 1, nucleotide 1132 to 474) into the EcoRV site of pBSIISK(+) SEQ ID NO: 6. Oligonucleotides 5'-ATATCATAATATGTACATTTATATTG-3', SEQ ID NO: 13 and 5'-TCGCGACGTCTCCGTGTAGGCGATCTGACGGTTCACTAAAC-3', SEQ ID NO:

14 were used to amplify the promoter.

The SV40 poly(A) signal, which can be obtained, e.g., from pCEP4, SEQ ID NO: 1 (from the native BsaBI site at nucleotide 176 to the native BamHI site at position 412) was cloned into the SmaI and BamHI sites of pBSSK(+)-CMVIE. Using the remaining EcoRI and PstI sites in between the CMV promoter and SV40 poly(A), a multicloning site was added using oligonucleotides:

5'-AATTCGCGACGCGTGATATCTGCAGGCCTAGATCTCTAGATAAGTAAT GATCATGCA-3', SEQ ID NO: 15

and

5'-TGATCATTACTTATCTAGAGATCTAGGCCTGCAGATATCACGCGTCGCG-3', SEQ ID NO: 2, yielding vector p394.

Vector p394 (Figure 1b), was cleaved with HindIII and BamHI to yield a 1.3 kb HindIII - BamHI fragment containing the cytomegalovirus immediate early promoter (CMV), a multicloning site region (mcs), and the SV40 poly(A) region. This fragment, which comprises an "expression cassette" was cloned into the HindIII and BamHI sites of pHEBo to yield pcmvmcs1 (Figure 1c). The mcs contains the following restriction enzyme sites: Esp3I, EcoRI, NruI, MluI, EcoRV, PstI, StuI, BglII. The mcs in vector pcmvmcs1 was replaced with the following sites: Esp3I, AgeI, StuI, KpnI, AvrII, XhoI, by a synthetic oligonucleotide linker that contained overhangs compatible with the Esp3I and BglII sites. The BglII site was not recreated by the oligonucleotide linker (Figure 2e). This vector was designated pcmvmcs3.

C. Construction of pm3ar Vector

An intron (called IVS or "intervening sequence") was added to the expression cassette (defined herein as the CMVIE-mcs-poly(A) containing nucleotides) as follows. A XhoI - BamHI fragment containing the SV40 early intron and poly(A) signals was excised from vector pCDM8 (Invitrogen, Carlsbad, CA; Figures 1d and 4), SEQ ID NO: 3. The poly(A)-containing fragment was removed from vector pcmvmcs3 by digestion with restriction enzymes XhoI and BamHI, and the XhoI-BamHI fragment from pCDM8 was added, generating vector pm3ar (Figure 1e).

D. CCR3 Expression Vector

An episomal vector which codes for the C-C chemokine receptor 3 ("CCR3") was constructed. The coding region for the receptor was obtained by PCR amplification of genomic DNA, using the oligonucleotide 5'-

GTGAAATGACAACCTCACTAGATACAG-3', SEQ ID NO: 4 as the sense primer, and 5'-CTGACCTAAAACACAATAGAGAGT-3', SEQ ID NO: 5, as the antisense primer. The PCR fragment obtained was cloned into the EcoRV site of pBSIISK+ (Figure 5)(a Bluescript vector commercially available from Stratagene, La Jolla, CA, Stratagene Cat. No. 212205, Genbank accession number 52325), SEQ ID NO. 6. The coding region was excised from pBSIISK+ using the restriction enzymes SpeI and NsiI, and the fragment containing DNA coding for CCR3 was cloned into the AvrII and Sse8387I sites of vector pm3ar (Figure 1e) to generate episomal expression construct pm3CCR3 (Figure 1f).

A hydrophobic signal sequence was added to the CCR3 coding sequence by PCR (see Figure 1g). Vector pm3CCR3 was used as a template and oligonucleotide 144, 5'-TGTCGATTGTCAGCAGGATTATG-3' SEQ ID NO: 7 (which begins at nucleotide +390 and maps 3' to the unique BglII restriction site on the vector) and oligonucleotide 143,5'-GTTCTGTCTCTGCTGCCACTG CTCGAGGCTCAAACAACCTCACTAGATACAGTTGAG-3', SEQ ID NO: 8 (which overlaps the CCR3 coding sequence and contains a long tail encoding approximately two-thirds of the hydrophobic signal sequence) were used as primers. The resulting 428 base pair fragment was then used as a template for PCR, using oligonucleotide 144, SEQ ID NO: 7 and oligonucleotide 142, GAGCAGCCGGCACC ACCATGGCTCTGTCTTGGGTTCTGACTGTTCTGTCTCTGCTGCCACTG, SEQ ID NO: 9 (which encodes the remainder of the hydrophobic signal sequence and contains a Kozak consensus sequence for efficient initiation of translation). The resulting 461 base pair fragment was digested with NgoMI and BglII and cloned into the AvrII and BglII sites of pm3CCR3 to generate expression vector pm3CCR3sp (Figure 1h).

E. Construction of pE3 Vector

Vector pm3ar (Figure 1e) was altered to provide an additional set of cloning sites immediately upstream from the CMVIE promoter. The new sites were added using a synthetic oligonucleotide linker 5'-CGATCACGTGCAGCTGAGATCTA-3', SEQ ID NO: 10, that contained the restriction sites, ClaI, AscI, BssHII, PacI, HindIII and overhangs compatible with the ClaI and HindIII sites of pm3ar. The new vector was designated pE3 (Figure 2a).

F. Construction of pE3delta Vector

Vector pE3delta (Figure 2b) was generated by the digestion of vector pE3 with BstBI and BspLU11I to remove the hygromycin coding region. The hygromycin coding region was replaced with a synthetic oligonucleotide linker 5'-CATGTAGATCTCAGCTGCACGTGAT-3', SEQ ID NO: 11 containing multiple cloning sites.

G. Construction of pE3pur Vector

Vector pE3pur (Figure 2d) was constructed by the digestion of vector pE3delta with PvuII and BspLU11I followed by ligation to a PvuII - BglIII fragment (Figure 2c) obtained from vector pPur (Clontech, Cat. No. 6156-1, Genbank accession number U07648, SEQ ID NO: 12, Figure 6). The PvuII - BglIII fragment from vector pPur contains the SV40 promoter, a puromycin resistance gene, and an SV40 poly(A) tail.

H. G_{ia2} Expression Vector

Vector pBN31, which contains the wildtype sequence for murine G_{ia2} cloned into the EcoRI site of vector pCDNAI, was obtained from the American Type Culture Collection (ATCC), Cat. No. 63311, Rockville, MD. pE3pur vector (Figure 2d) was digested with KpnI and XhoI, which correspond to restriction sites found within the multicloning regions at the 5' and 3' ends, respectively, of the G_{ia2} coding region. The vector obtained from the ATCC was also digested with KpnI and XhoI, and a fragment containing the G_{ia2} coding region was excised. This fragment was cloned into the KpnI and XhoI sites of vector pE3pur, to produce vector pEpurGia2 (Figure 2e). This vector was used without further modification to transfect cells.

EXAMPLE 2: TRANSFECTION OF CELLS AND MAINTENANCE OF STABLY TRANSFECTED CELL LINES

293E cells (HEK-293 cells which constitutively express the Epstein-Barr virus nuclear antigen-1, commercially available from Invitrogen Corp., Carlsbad, CA, cat. no. R620-07) were transfected using the calcium phosphate or lipofectamine procedures as described in Sambrook et al., (1989) "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1633-1634.

T75 flasks containing approximately 1 x 10^6 293E cells were transfected with plasmid p3ARccr3 with or without cotransfection with pEpurGia2. 5 mg of each plasmid was used in the transfection reaction, with 62 ml of 2 M $CaCl_2$ in enough

water to achieve a final volume of 500 ml. To this solution was added 500 ml of HEPES-buffered saline ("HBS") and the entire 1 ml of solution was added directly to the T75 culture medium (12 ml of Dulbecco's Modified Eagle Medium, "DMEM", supplemented with 10% fetal bovine serum, penicillin 100 units/ml, streptomycin 100 mg/ml, glutamine 2 mM, hereinafter called "complete DMEM"). The transfection mix was allowed to remain on the cells for 48 hours at which time the cells were washed once with phosphate buffered saline and re-fed selective media (complete DMEM supplemented with 1 mg/ml puromycin and 250 mg/ml hygromycin, hereinafter called "selective media"). The selective media was changed at day 5 post-transfection (i.e., day 5 after the transfection mix was removed) and again when the cells approached confluence, at which time cells were routinely split, i.e., "passed" between 1:4 and 1:50 in fresh selective media as needed. Frozen stocks of cells were made at cell passage 3.

EXAMPLE 3: STABILITY OF EXPRESSION FROM EPISOMES

Cells expressing either the CCR3 receptor, or both the CCR3 receptor and G_{ia2} protein, generated according to Example 2, were assayed for agonist binding characteristics. The cells were incubated in ^{125}I-eotaxin at concentrations ranging from 45 pM to 300 nM. Specific binding was determined at each concentration, as was the maximum binding for a given number of cells. Based on these values, the Kd for eotaxin was determined at the end of 1 to 5 months after vector transfection. As can be seen from Figure 7, the Kd for the cells was stable, remaining between 0.2 and 0.3 nM for the entire five months for the cotransfected cells, and between 0.2 and 0.5 nM for the cells containing CCR3 receptors which were not coexpressed with G_{ia2}.

In addition to the stability observed for Kd values, the total receptor number was also stable over the five month period, regardless of whether the CCR3 receptor was coexpressed with G_{ia2} protein or not. In particular, Figure 8 shows that the receptor number in the singly transfected and cotransfected cells was approximately equal and stable over the entire 5 month assay period, at about 20,000 receptors per cell.

The stability of the Kd of the receptors for agonist over time is important, as variations in Kd, particularly increases in Kd, could lead to false negative responses in assays for compounds that bind to receptors.

EXAMPLE 4: ASSAYS FOR CALCIUM MOBILIZATION IN RESPONSE TO RECEPTOR BINDING OF AGONIST COMPOUNDS

Assays were conducted utilizing the methods described in S.R. McColl and R.H. Naccache, *Methods in Enzymology* 288:301-309, 1997, "Calcium Mobilization Assays". Cell suspensions (10^7 cells/ml) were loaded with the fluorescent probe Fluo-3/AM (Teflabs, Texas) at 2 mM for 60 minutes at room temperature. The cells were washed, resuspended at 2-4 x 10^6 cells/ml in Hanks' Buffered Salt Solution, made 1.6 mM in $CaCl_2$ and 10 mM in HEPES (HBSS). Mock transfected cells, cells transfected with the CCR3 receptor only, cells transfected with G_{ia2} protein only, and cells cotransfected with CCR3 receptor and with G_{ia2} protein were then incubated in medium which comprised 100 nM of the CCR3 receptor agonist eotaxin. The cells' fluorescence was measured in a spectrofluorometer (Model SLM, Bowman Series 2, SLM-Aminco, Champagne, IL) at both the excitation wavelength, i.e., 506 nm, and the emission wavelength, i.e., 526 nm, for the chromophore. Internal calcium concentrations were calculated as described in Tsien, et al., *J. Cell. Biol.* 94:3325 (1982). Each assay was individually calibrated.

Upon addition of receptor agonist, a nearly four-fold increase in fluorescence was detected in the cells which co-expressed the CCR3 receptor and G_{ia2} protein compared to the fluorescence achieved in the cells transfected with the CCR3 receptor alone (Figure 9). No calcium increases were observed in mock-transfected cells or in cells transfected with the G-proteins alone upon stimulation with eotaxin. The data demonstrate that the episomally expressed receptor/G-protein alpha subunit signal transduction apparatus is useful as early as two weeks post transfection, and is stable for at least 20 weeks.

A similar experiment was performed using the nociceptin receptor. The results shown in Figure 10 demonstrate that upon addition of nociceptin receptor agonist, a two-fold increase in fluorescence was detected in the cells which co-expressed the nociceptin receptor and G_{ia2} protein compared to the fluorescence achieved in the cells transfected with the nociceptin receptor alone. Again, receptor and G protein alpha subunit expression from episomes provided for readily assayable calcium responses in as little as two weeks.

Figure 11 shows the increase in calcium flux that can be obtained in cells cotransfected with the CCR2 receptor and G_{ia2} relative to the calcium release

obtainable in cells transfected with only the CCR2 receptor in response to the chemokine MCP-1 (30 nM). The experiment was performed 2 months after transfection or cotransfection of cells. As can be seen from Figure 11, calcium release in cotransfected cells in response to exposure to CCR2 agonist MCP-1 was significantly greater than in cells which only expressed the CCR2 receptor.

Calcium release in response to increasing concentrations of interleukin 8 was evaluated in cells expressing either the interleukin 8 receptor type B alone, or the interleukin 8 receptor type B coexpressed with G_{ia2}. The cells had been transfected 1 month prior to the assay. As can be seen from the data presented in Figure 12, there was an increase in the amount of calcium released from the cotransfected cells relative to the calcium released by the cells which were transfected only with the interleukin 8 receptor type B. These data are consistent with those presented for the CCR3, CCR2, and nociceptin receptors.

Thus, these data demonstrate that coexpression of G_i alpha proteins with G_i protein coupled receptors provides for consistent increases in calcium signaling, allowing for more sensitive and reliable assays for receptor binding compounds, i.e., agonists and antagonists.

WHAT IS CLAIMED IS:

1. A method for assaying a test compound for its effect on a G_i protein coupled receptor which comprises the steps of:

contacting said cell with a test compound; and

assaying said cell for calcium concentration in the cytoplasm of said cell;

said cell having been transfected with a gene encoding a G_i protein coupled receptor and with a gene coding for G_{ia} protein capable of coupling to said G_i protein coupled receptor to increase said cytoplasmic calcium upon binding of an agonist to said G_i protein coupled receptor.

2. The method of claim 1 wherein said test compound is a receptor antagonist.

3. The method of claim 1 wherein said test compound is a receptor agonist.

4. The method of claim 1 wherein said gene coding for a G_{ia} protein codes for G_{ia2}.

5. The method of claim 1 wherein said gene coding for a G_{ia} protein codes for G_{ia1} or G_{ia3}.

6. The method of claim 1 wherein said gene coding for a G_i protein coupled receptor and said gene coding for a G_{ia} protein capable of coupling to said receptor are expressed from separate DNA constructs.

7. The method of claim 1 wherein said gene coding for a G_i protein coupled receptor and said gene coding for a G_{ia} protein capable of coupling to said receptor are expressed from a single DNA construct.

8. The method of claim 1 comprising direct measurement of said cytoplasmic calcium concentration within said cell.

9. The method of claim 1 comprising indirect measurement of said cytoplasmic calcium concentration within said cell.

10. The method of claim 1 wherein said gene coding for said G_i protein coupled receptor is integrated in chromosomal DNA of said cell.

11. The method of claim 1 wherein said gene coding for said G_i protein coupled receptor is expressed by a stably transfected episomal expression vector.

12. The method of claim 1 wherein said gene coding for said G_i protein coupled receptor is expressed by a transiently transfected expression vector.

13. The method of claim 1 wherein said gene coding for said G_{ia} protein is integrated in said native DNA of said cell.

14. The method of claim 1 wherein said gene coding for said G_{ia} protein is expressed by a stably transfected episomal expression vector.

15. The method of claim 1 wherein said gene coding for said G_{ia} protein is expressed by a transiently transfected expression vector.

16. The method of claim 11 wherein said gene coding for said G_{ia} protein is expressed by a stably transfected episomal expression vector.

17. The method of claim 16 wherein said G_i protein coupled receptor is selected from the group consisting of the C-C chemokine receptor 3, the C-C chemokine receptor 2, the interleukin 8 type B receptor, and the nociceptin receptor.

18. The method of claim 16 wherein said assaying comprises:

detecting fluorescence produced by said cell;

said cell having been loaded with a fluorophore that fluoresces in the presence of calcium.

19. The method of claim 18 wherein said fluorophore is Fura-2/AM, Indo-1/AM, or Fluo-3/AM.

20. The method of claim 1 wherein said cell has not also been transfected with genes encoding $G_{i\beta}/G_{ig}$ dimer or phospholipase C ß.

21. The method of claim 1 wherein said test compound is a receptor antagonist and is contacted with said cell in the presence of a receptor agonist, and wherein said step of contacting decreases said calcium concentration a compared with the calcium concentration of said cells contacted with said receptor agonist alone.

22. A cell useful for assaying a test compound for its effect on a G_i protein coupled receptor, said cell comprising:

transfected DNA which expresses a G_i coupled receptor;

transfected DNA which expresses a G_{ia} protein subunit; and

an intracellular calcium release mechanism which produces a detectable signal as measured by fluorescence of a fluorophore in response to agonist compound binding to said receptor when said G_i protein coupled receptor is coexpressed with said G_{ia} protein subunit.

23. The cell of claim 22 wherein said DNA which expresses a G_i coupled receptor is exogenous DNA that has been stably integrated into chromosomal DNA of said cell.

24. The cell of claim 22 wherein said DNA which expresses a G_i coupled receptor is contained in a stably transfected episomal expression vector.

25. The cell of claim 22 wherein said DNA which expresses a G_i coupled receptor is contained in a transiently transfected episomal expression vector.

26. The cell of claim 22 wherein said DNA which expresses a G_{ia} protein subunit is exogenous DNA stably integrated into chromosomal DNA of said cell.

27. The cell of claim 22 wherein said DNA which expresses a G_{ia} protein subunit is contained in a stably transfected episomal expression vector.

28. The cell of claim 22 wherein said DNA which expresses a G_{ia} protein subunit is contained in a transiently transfected expression vector.

29. The cell of claim 22 wherein said G_i protein coupled receptor is selected from the group consisting of the C-C chemokine receptor 3, the C-C chemokine receptor 2, the interleukin 8 type B receptor, and the nociceptin receptor.

30. The cell of claim 22 wherein said fluorophore is Fura-2/AM, Indo-1/AM, or Fluo-3/AM.

31. The cell of claim 22 which has not also been transfected with genes encoding $G_{i\beta}/G_i$ dimer or phospholipase C ß.

32. A method for producing a cell for detecting compounds which bind to a G_i protein coupled receptor which comprises the steps of:

transfecting a cell with a gene which codes for a G_i protein coupled receptor;

transfecting said cell with a gene which codes for a G_{ia} protein capable of coupling to said receptor; and

culturing said cell in medium under conditions in which the G_{ia} protein and the G_i protein coupled receptor are co-expressed in said cell.

33. The method of claim 32 wherein said gene coding for a G_i protein coupled receptor and said gene coding for a G_{ia} protein capable of coupling to said receptor are each expressed from a different DNA construct.

34. The method of claim 32 wherein said gene coding for a G_{ia} protein codes for a member selected from the group consisting of G_{ia1} and G_{ia3}.

35. The method of claim 32 wherein said gene coding for a G_{ia}

protein codes for G_{ia2}.

FIG. 1a

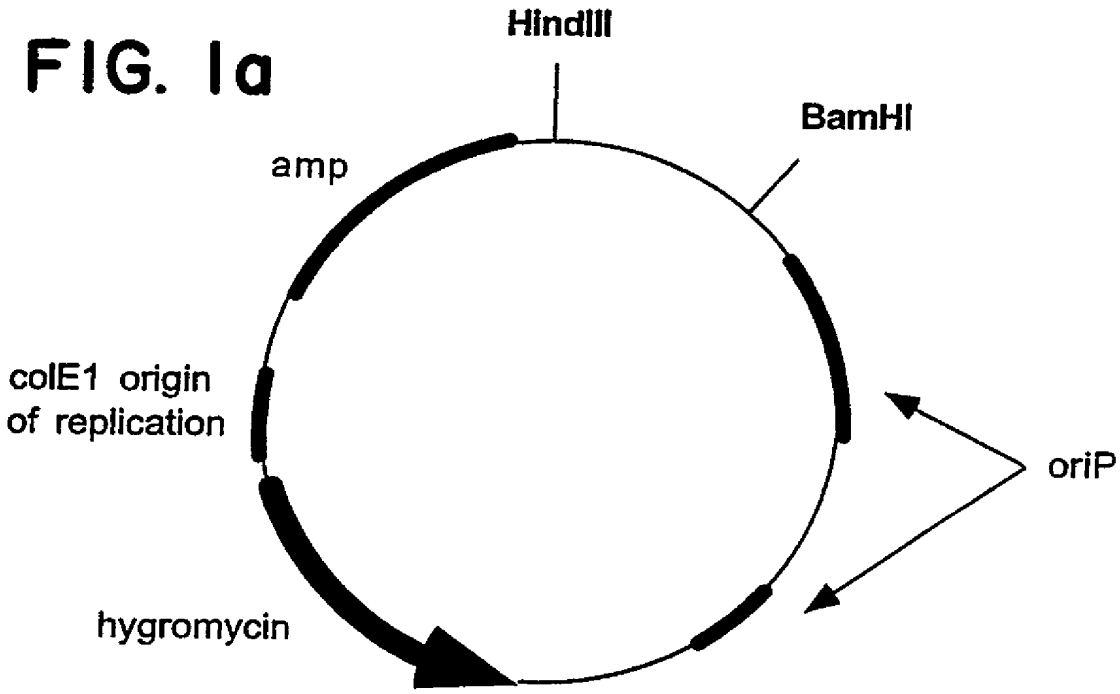
HindIII
BamHI
amp
colE1 origin
of replication
oriP
hygromycin

FIG. 1b

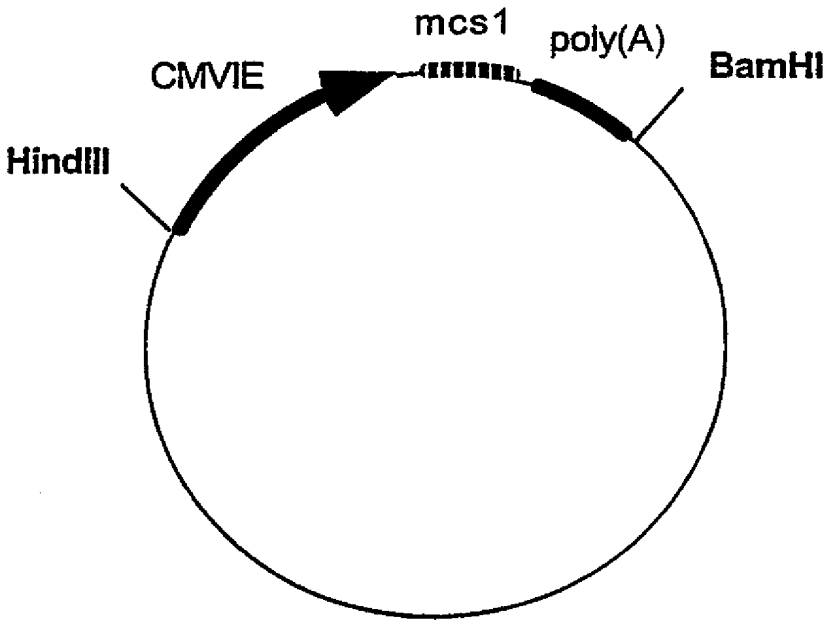
mcs1
poly(A)
CMVIE
BamHI
HindIII

FIG. 1c

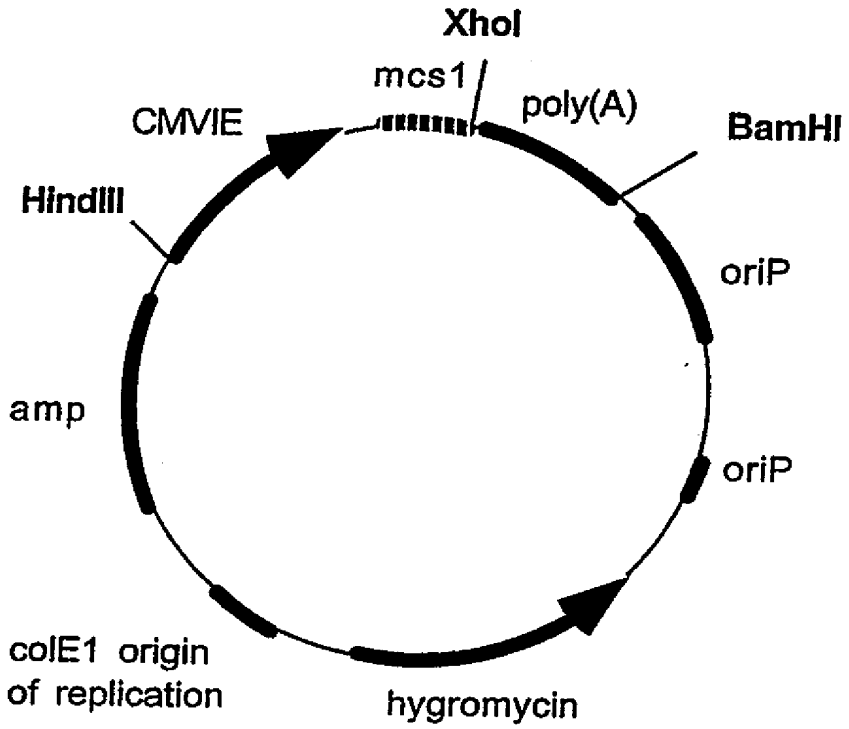

FIG. 1d

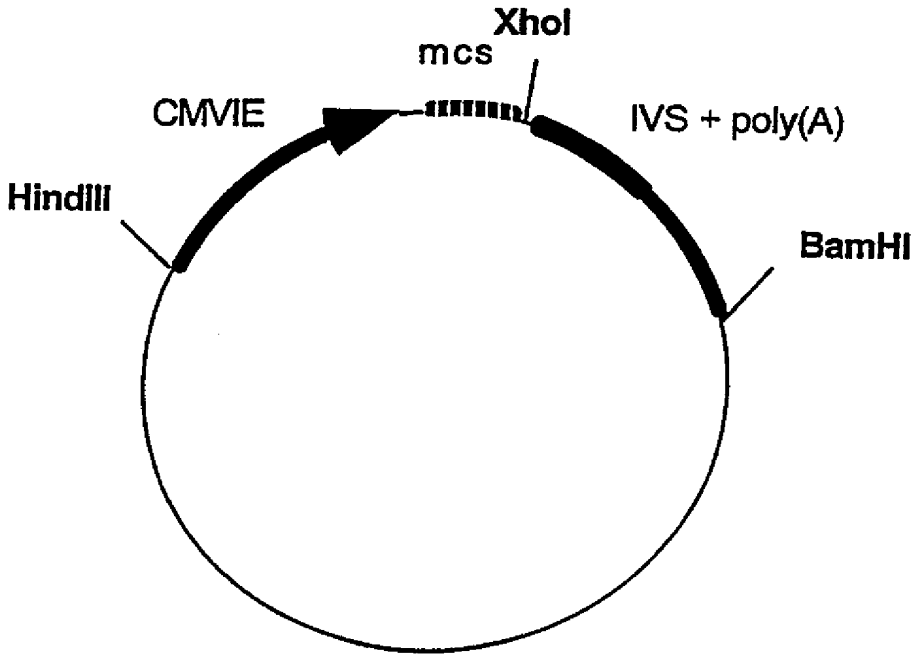

FIG. 1e

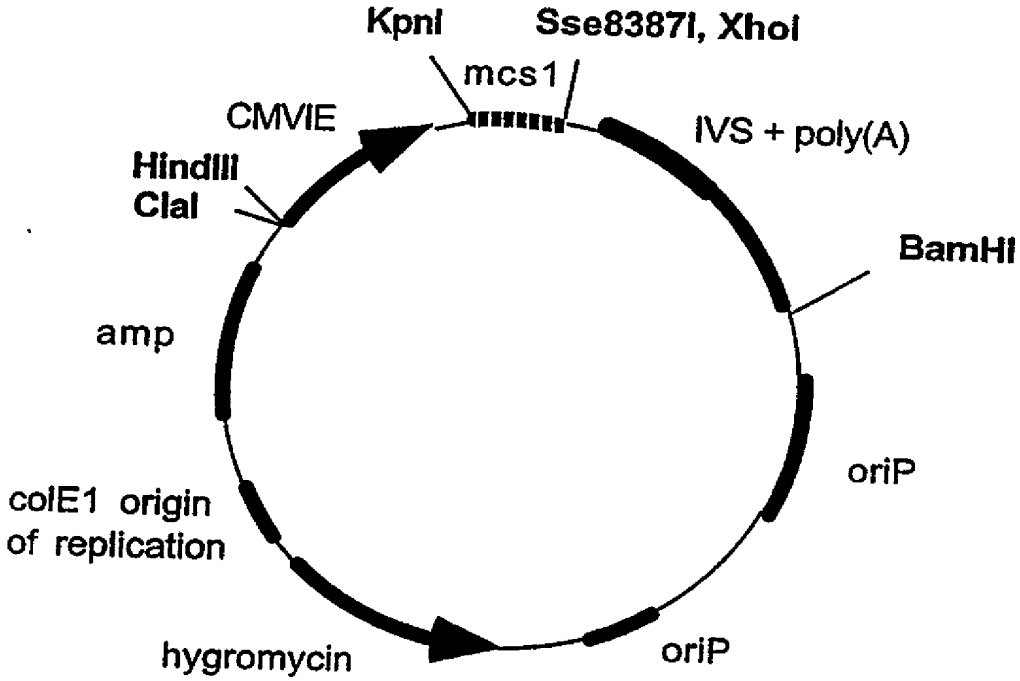

FIG. 1f

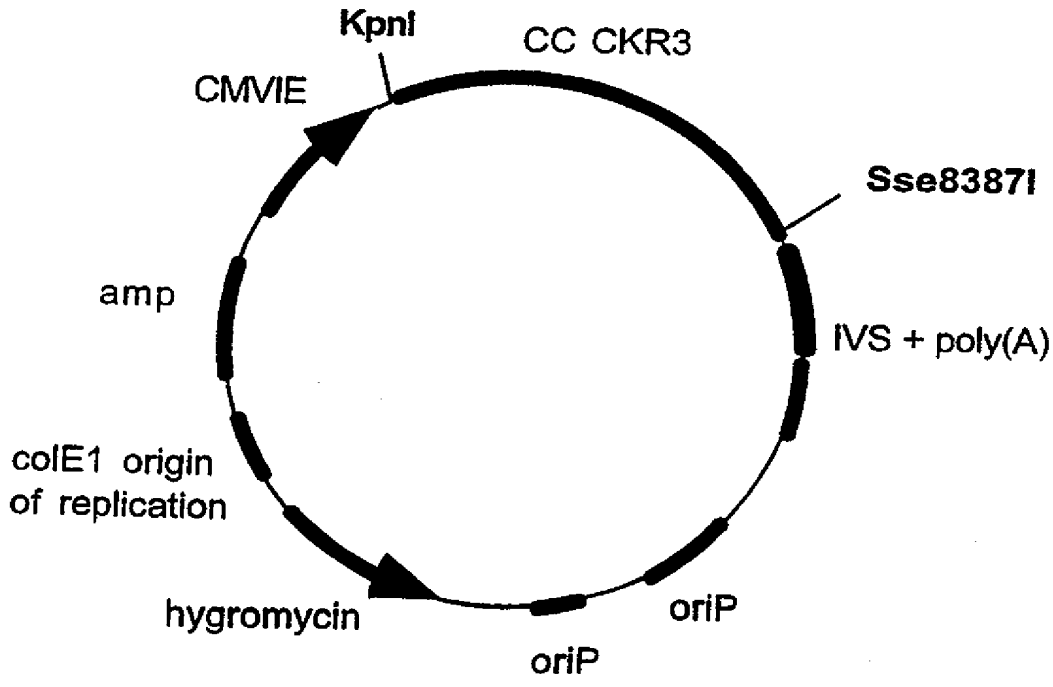

FIG. 1g

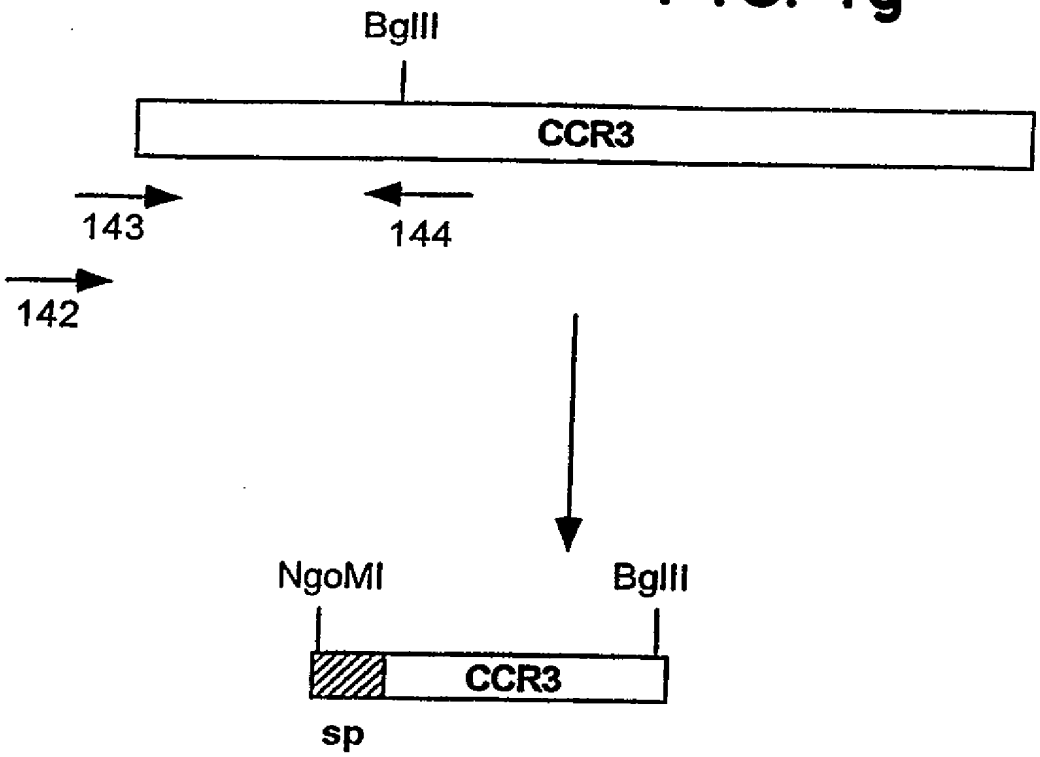

FIG. 1h

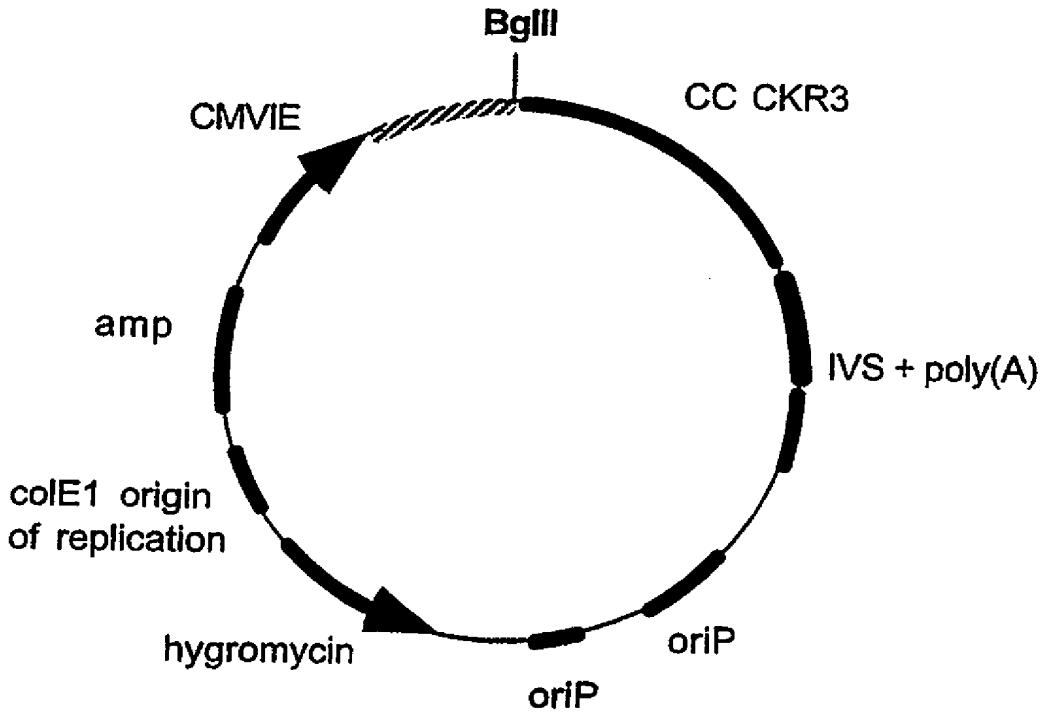

FIG. 2a

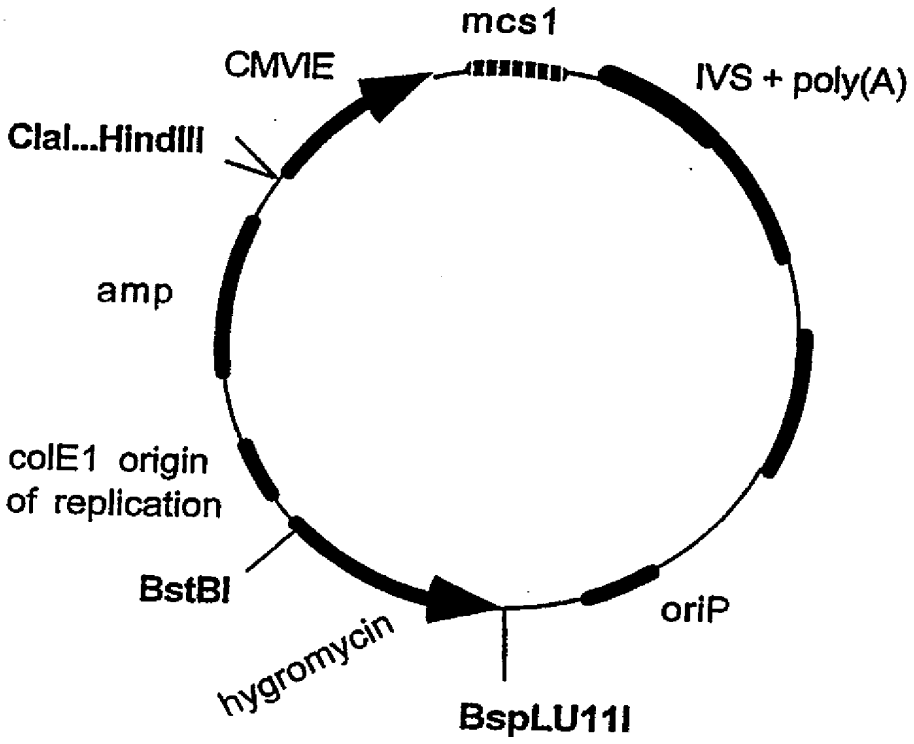

FIG. 2b

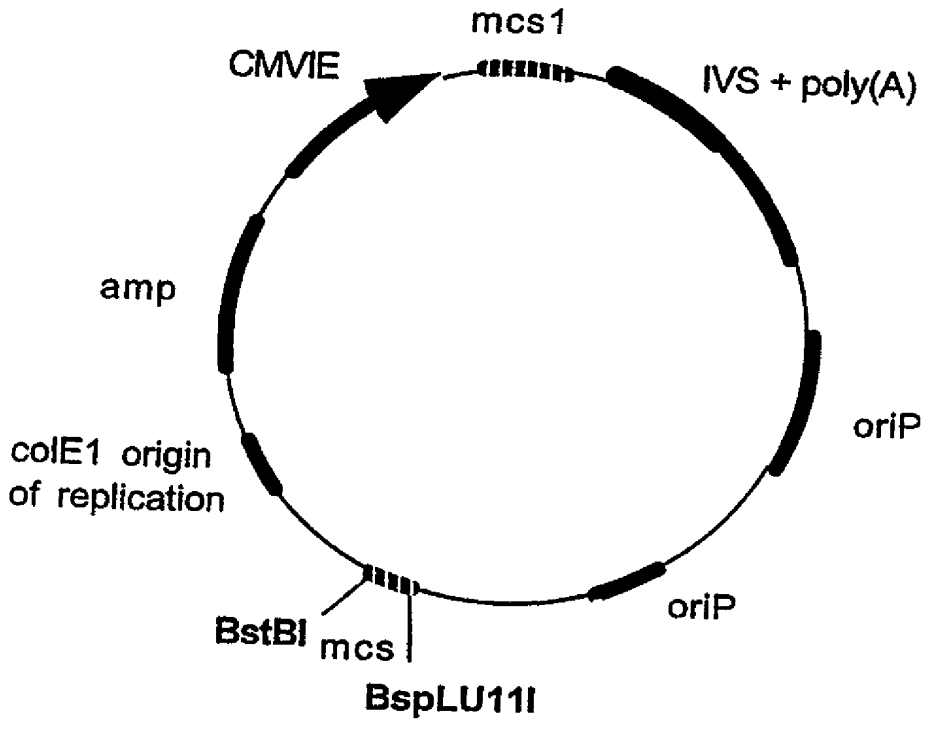

FIG. 2c

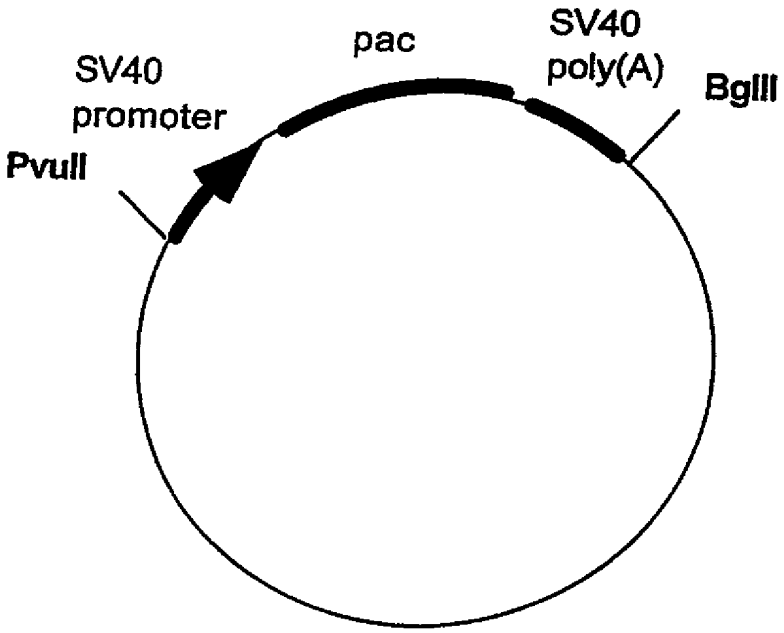

FIG. 2d

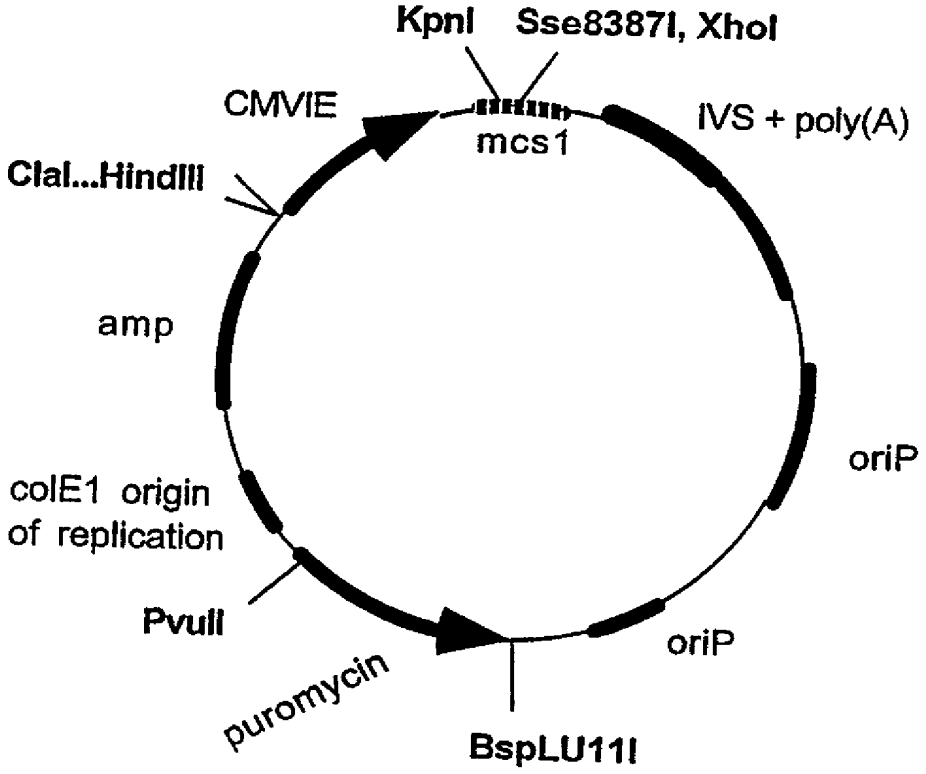

FIG. 2e

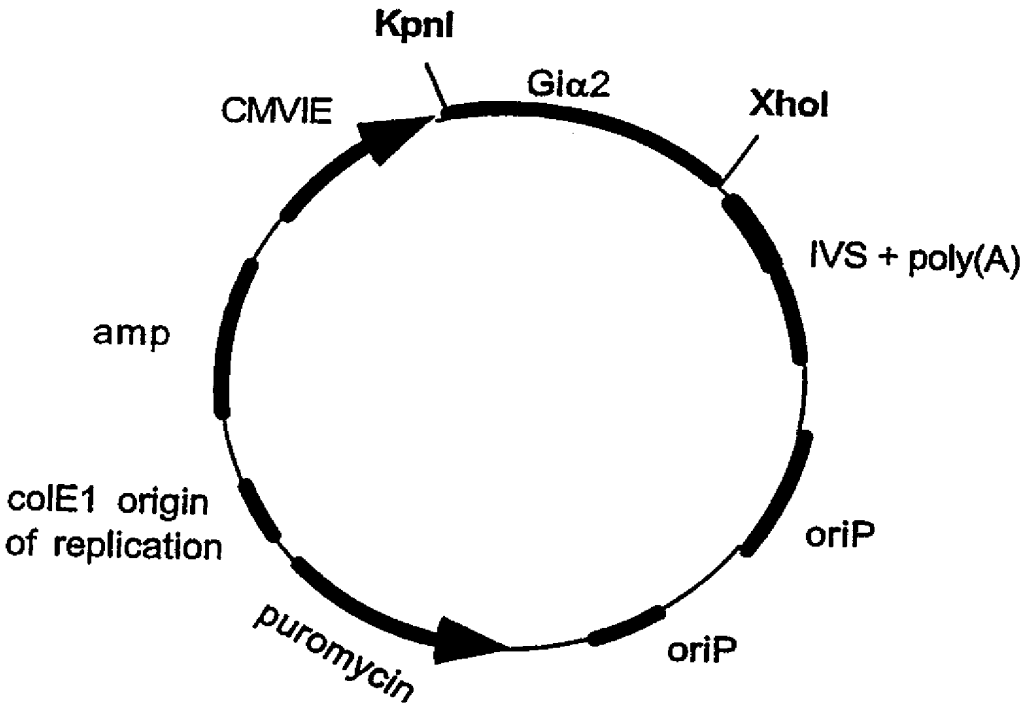

FIG. 3a

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 101 TGTGACCGTC TCCGGGAGCT GCATGTGTCA GAGGTTTTCA CCGTCATCAC
 151 CGAAACGCGC GAGGCAGCCG GATCATAATC AGCCATACCA CATTTGTAGA
 201 GGTTTTACTT GCTTTAAAAA ACCTCCCCAC CTCCCCCTGA ACCTGAAACA
 251 TAAAATGAAT GCAATTGTTG TTGTTAACTT GTTTATTGCA GCTTATAATG
 301 GTTACAAATA AAGCAATAGC ATCACAAATT TCACAAATAA AGCATTTTTT
 351 TCACTGCATT CTAGTTGTGG TTTGTCCAAA CTCATCAATG TATCTTATCA
 401 TGTCTGGATC CGGCCTTGCC GGCCTCGAGC GGCCGCTAGC AAGCTTGCTA
 451 GCAGCTGGTA CCCAGCTTCT AGAGATCTGA CGGTTCACTA AACGAGCTCT
 501 GCTTATATAG ACCTCCCACC GTACACGCCT ACCGCCCATT TGCGTCAACG
 551 GGGCGGGGTT ATTACGACAT TTTGGAAAGT CCCGTTGATT TTGGTGCCAA
 601 AACAAACTCC CATTGACGTC AATGGGGGTGG AGACTTGGAA ATCCCCGTGA
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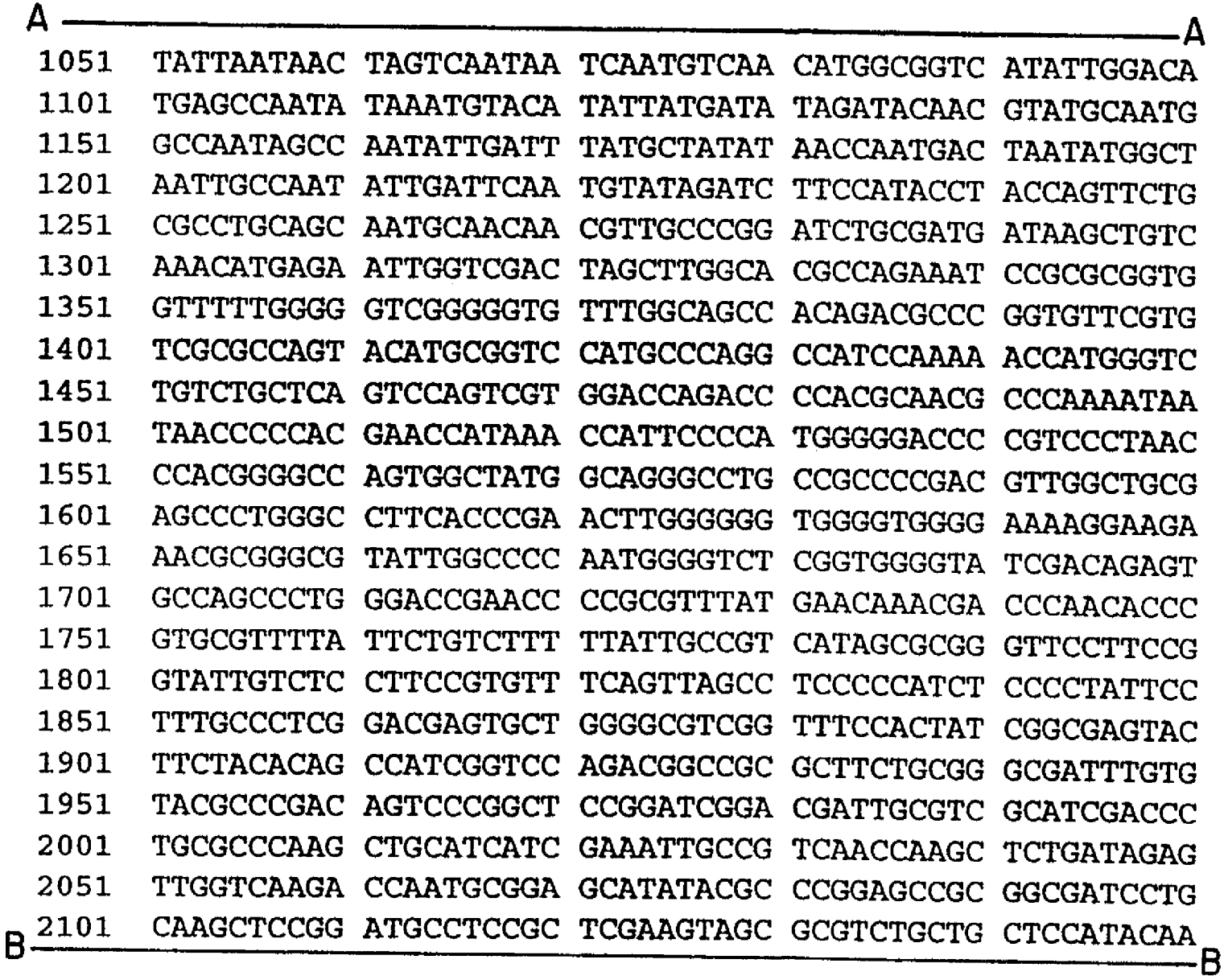
 701 TGGTAATAGC GATGACTAAT ACGTAGATGT ACTGCCAAGT AGGAAAGTCC
 751 CGTAAGGTCA TGTACTGGGC ATAATGCCAG GCGGGCCATT TACCGTCATT
 801 GACGTCAATA GGGGGCGGAC TTGGCATATG ATACACACTTGA TGTACTGCCA
 851 AGTGGGCAGT TTACCGTAAA TACTCCACCC ATTGACGTCA ATGGAAAGTC
 901 CCTATTGGCG TTACTATGGG AACATACGTC ATTATTGACG TCAATGGGCG
 951 GGGGTCGTTG GGCGGTCAGC CAGGCGGGCC ATTTACCGTA AGTTATGTAA
1001 CGCGGAACTC CATATATGGG CTATGAACTA ATGACCCCGT AATTGATTAC
   A ——————————————————————————————————————— A
```

FIG. 3b


A
A
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1101 TGAGCCAATA TAAATGTACA TATTATGATA TAGATACAAC GTATGCAATG
1151 GCCAATAGCC AATATTGATT TATGCTATAT AACCAATGAC TAATATGGCT
1201 AATTGCCAAT ATTGATTCAA TGTATAGATC TTCCATACCT ACCAGTTCTG
1251 CGCCTGCAGC AATGCAACAA CGTTGCCCGG ATCTGCGATG ATAAGCTGTC
1301 AAACATGAGA ATTGGTCGAC TAGCTTGGCA CGCCAGAAAT CCGCGCGGTG
1351 GTTTTTGGGG GTCGGGGGTG TTTGGCAGCC ACAGACGCCC GGTGTTCGTG
1401 TCGCGCCAGT ACATGCGGTC CATGCCCAGG CCATCCAAAA ACCATGGGTC
1451 TGTCTGCTCA GTCCAGTCGT GGACCAGACC CCACGCAACG CCCAAAATAA
1501 TAACCCCCAC GAACCATAAA CCATTCCCCA TGGGGGACCC CGTCCCTAAC
1551 CCACGGGGCC AGTGGCTATG GCAGGGCCTG CCGCCCCGAC GTTGGCTGCG
1601 AGCCCTGGGC CTTCACCCGA ACTTGGGGGG TGGGGTGGGG AAAAGGAAGA
1651 AACGCGGGCG TATTGGCCCC AATGGGGTCT CGGTGGGGTA TCGACAGAGT
1701 GCCAGCCCTG GGACCGAACC CCGCGTTTAT GAACAAACGA CCCAACACCC
1751 GTGCGTTTTA TTCTGTCTTT TTATTGCCGT CATAGCGCGG GTTCCTTCCG
1801 GTATTGTCTC CTTCCGTGTT TCAGTTAGCC TCCCCCATCT CCCCTATTCC
1851 TTTGCCCTCG GACGAGTGCT GGGGCGTCGG TTTCCACTAT CGGCGAGTAC
1901 TTCTACACAG CCATCGGTCC AGACGGCCGC GCTTCTGCGG GCGATTTGTG
1951 TACGCCCGAC AGTCCCGGCT CCGGATCGGA CGATTGCGTC GCATCGACCC
2001 TGCGCCCAAG CTGCATCATC GAAATTGCCG TCAACCAAGC TCTGATAGAG
2051 TTGGTCAAGA CCAATGCGGA GCATATACGC CCGGAGCCGC GGCGATCCTG
2101 CAAGCTCCGG ATGCCTCCGC TCGAAGTAGC GCGTCTGCTG CTCCATACAA
B
B

FIG. 3c

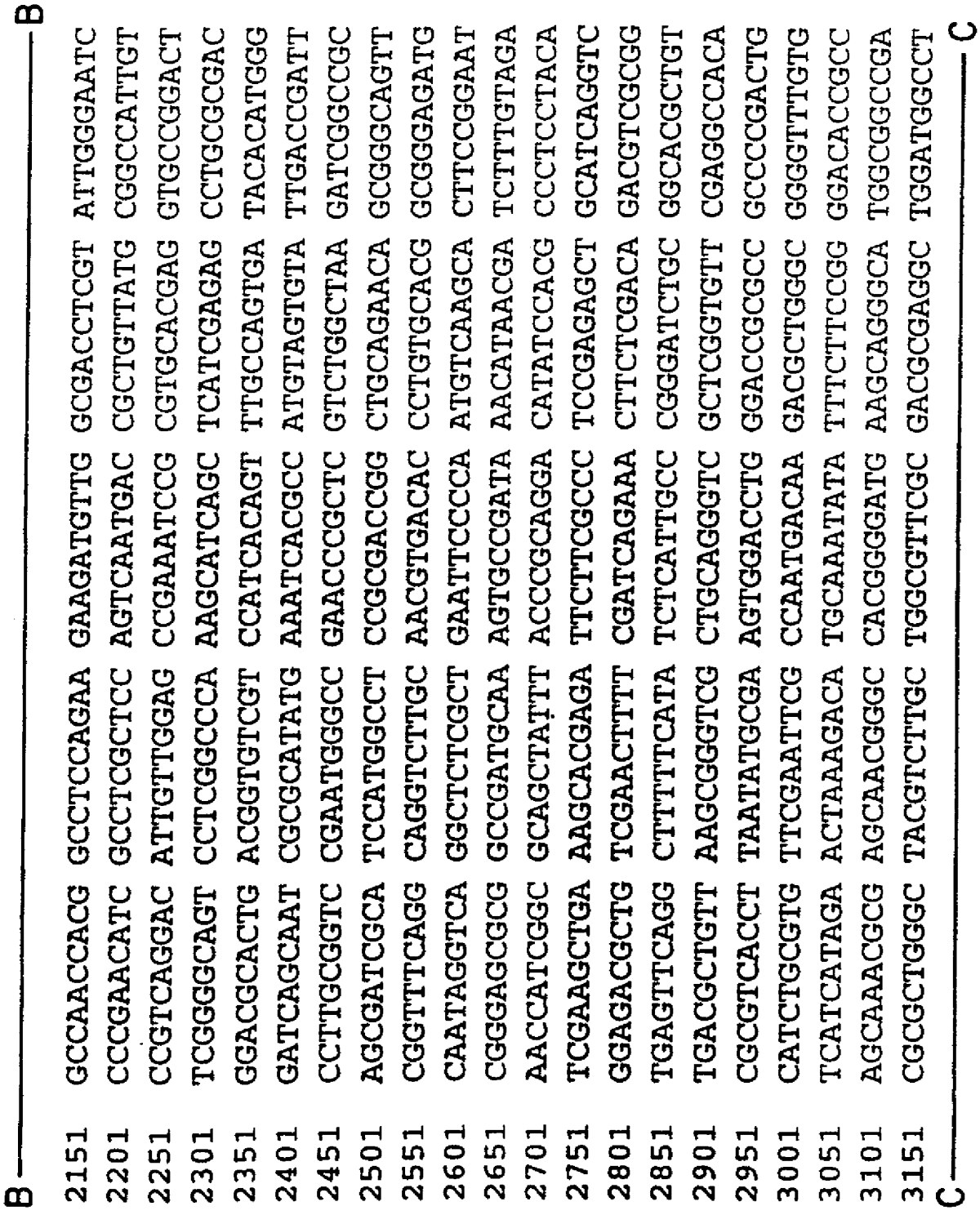


FIG. 3d

C

```
3201  TCCCCATTAT GATTCTTCTC GCTTCCGGCG GCATCGGGAT GCCCGCGTTG
3251  CAGGCCATGC TGTCCAGGCA GGTAGATGAC GACCATCAGG GACAGCTTCA
3301  AGGATCGCTC GCGGCTCTTA CCAGCCTAAC TTCGATCACT GGACCGCTGA
3351  TCGTCACGGC GATTTATGCC GCCTCGGCGA GCACATGGAA CGGGTTGGCA
3401  TGGATTGTAG GCGCCGCCCT ATACCTTGTC TGCCTCCCCG CGTTGCGTCG
3451  CGGTGCATGG AGCCGGGCCA CCTCGACCTG AATGGAAGCC GGCGGCACCT
3501  CGCTAACGGA TTCACCACTC CAAGAATTGG AGCCAATCAA TTCTTGCGGA
3551  GAACTGTGAA TGCGCAAACC AACCCTTGGC AGAACATATC CATCGCGTCC
3601  GCCATCTCCA GCAGCCGCAC GCGGCGCAGC AAAAGGCCAG GAACCGTAAA
3651  AAGGCCGCGT TGCTGGCGTT TTTCCATAGG CTCCGCCCCC CTGACGAGCA
3701  TCACAAAAAT CGACGCTCAA GTCAGAGGTG GCGAAACCCG ACAGGACTAT
3751  AAAGATACCA GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT
3801  CCGACCCTGC CGCTTACCGG ATACCTGTCC GCCTTTCTCC CTTCGGGAAG
3851  CGTGGCGCTT TCTCATAGCT CACGCTGTAG GTATCTCAGT TCGGTGTAGG
3901  TCGTTCGCTC CAAGCTGGGC TGTGTGCACG AACCCCCCGT TCAGCCCGAC
3951  CGCTGCGCCT TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA
4001  CGACTTATCG CCACTGGCAG CAGCCACTGG TAACAGGATT AGCAGAGCGA
4051  GGTATGTAGG CGGTGCTACA GAGTTCTTGA AGTGGTGGCC TAACTACGGC
4101  TACACTAGAA GGACAGTATT TGGTATCTGC GCTCTGCTGA AGCCAGTTAC
4151  CTTCGGAAAA AGAGTTGGTA GCTCTTGATC CGGCAAACAA ACCACCGCTG
4201  GTAGCGGTGG TTTTTTTGTT TGCAAGCAGC AGATTACGCG CAGAAAAAAA
4251  GGATCTCAAG AAGATCCTTT GATCTTTTCT ACGGGGTCTG ACGCTCAGTG
```

D

FIG. 3e

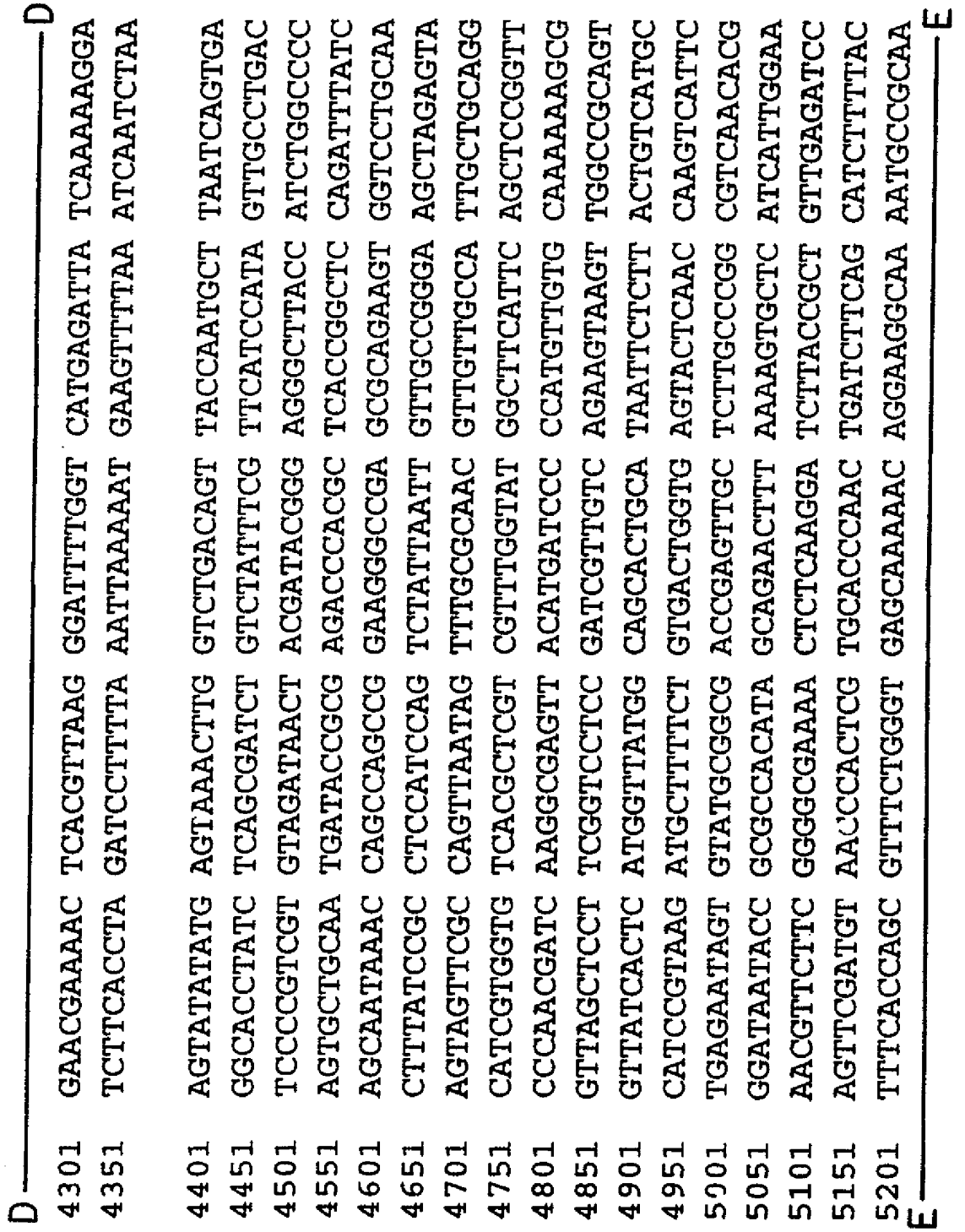


FIG. 3f

E

```
5251  AAAAGGGAAT AAGGGCGACA CGGAAATGTT GAATACTCAT ACTCTTCCTT
5301  TTTCAATATT ATTGAAGCAT TTATCAGGGT TATTGTCTCA TGAGCGGATA
5351  CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCGCACAT
5401  TTCCCCGAAA AGTGCCACCT GACGTCTAAG AAACCATTAT TATCATGACA
5451  TTAACCTATA AAAATAGGCG TATCACGAGG CCCTTTCGTC TTCAAGAATT
5501  CTCATGTTTG ACAGCTTATC ATCGATAAGC TGATCCTCAC AGGCCGCACC
5551  CAGCTTTTCT TCCGTTGCCC CAGTAGCATC TCTGTCTGGT GACCTTGAAG
5601  AGGAAGAGGA GGGGTCCCGA GAATCCCCAT CCCTACCGTC CAGCAAAAAG
5651  GGGGACGAGG AATTTGAGGC CTGGCTTGAG GCTCAGGACG CAAATCTTGA
5701  GGATGTTCAG CGGGAGTTTT CCGGGCTGCG AGTAATTGGT GATGAGGACG
5751  AGGATGGTTC GGAGGATGGG GAATTTTCAG ACCTGGATCT GTCTGACAGC
5801  GACCATGAAG GGGATGAGGG TGGGGGGGCT GTTGGAGGGG GCAGGAGTCT
5851  GCACTCCCTG TATTCACTGA GCGTCGTCTA ATAAAGATGT CTATTGATCT
5901  CTTTTAGTGT GAATCATGTC TGACGAGGGG CCAGGTACAG GACCTGGAAA
5951  TGGCCTAGGA GAGAAGGGAG ACACATCTGG ACCAGAAGGC TCCGGCGGCA
6001  GTGGACCTCA AAGAAGAGGG GGTGATAACC ATGGACGAGG ACGGGGAAGA
6051  GGACGAGGAC GAGGAGGCGG AAGACCAGGA GCCCCGGGCG GCTCAGGATC
6101  AGGGCCAAGA CATAGAGATG GTGTCCGGAG ACCCCAAAAA CGTCCAAGTT
6151  GCATTGGCTG CAAAGGGACC CACGGTGGAA CAGGAGCAGG AGCAGGAGCG
6201  GGAGGGGCAG GAGCAGGAGG GGCAGGAGCA GGAGGAGGGG CAGGAGCAGG
6251  AGGAGGGGCA GGAGGGGCAG GAGGGGCAGG AGGGGCAGGA GCAGGAGGAG
6301  GGGCAGGAGC AGGAGGAGGG GCAGGAGGGG CAGGAGGGGC AGGAGCAGGA
6351  GGAGGGGCAG GAGCAGGAGG AGGGGCAGGA GGGGCAGGAG CAGGAGGAGG
```

F F

FIG. 3g

F

6401	GGCAGGAGGG	GCAGGAGGGG	CAGGAGCAGG	AGGAGGGGCA	GGAGCAGGAG
6451	GAGGGGCAGG	AGGGGCAGGA	GCAGGAGGAG	GGGCAGGAGG	GGCAGGAGGG
6501	GCAGGAGCAG	GAGGAGGGGC	AGGAGCAGGA	GGGGCAGGAG	GGGCAGGAGG
6551	GGCAGGAGCA	GGAGGGGCAG	GAGCAGGAGG	AGGGGCAGGA	GGGGCAGGAG
6601	GGGCAGGAGC	AGGAGGGGCA	GGAGCAGGAG	GGGCAGGAGC	AGGAGGGGCA
6651	GGAGCAGGAG	GGGCAGGAGG	GGCAGGAGCA	GGAGGGGCAG	GAGGGGCAGG
6701	AGCAGGAGGG	GCAGGAGGGG	CAGGAGCAGG	AGGAGGGGCA	GGAGGGGCAG
6751	GAGCAGGAGG	AGGGGCAGGA	GGGGCAGGAG	CAGGAGGGGC	AGGAGGGGCA
6801	GGAGCAGGAG	GGGCAGGAGG	GGCAGGAGCA	GGAGGGGCAG	GAGGGGCAGG
6851	AGCAGGAGGA	GGGGCAGGAG	CAGGAGGGGC	AGGAGCAGGA	GGTGGAGGCC
6901	GGGGTCGAGG	AGGCAGTGGA	GGCCGGGGTC	GAGGAGGTAG	TGGAGGCCGG
6951	GGTCGAGGAG	GTAGTGGAGG	CCGCCGGGGT	AGAGGACGTG	AAAGAGCCAG
7001	GGGGGGAAGT	CGTGAAAGAG	CCAGGGGGAG	AGGTCGTGGA	CGTGGAGAAA
7051	AGAGGCCCAG	GAGTCCCAGT	AGTCAGTCAT	CATCATCCGG	GTCTCCACCG
7101	CGCAGGCCCC	CTCCAGGTAG	AAGGCCATTT	TTCCACCCTG	TAGGGGAAGC
7151	CGATTATTTT	GAATACCACC	AAGAAGGTGG	CCCAGATGGT	GAGCCTGACG
7201	TGCCCCCGGG	AGCGATAGAG	CAGGGCCCCG	CAGATGACCC	AGGAGAAGGC
7251	CCAAGCACTG	GACCCCGGGG	TCAGGGTGAT	GGAGGCAGGC	GCAAAAAAGG
7301	AGGGTGGTTT	GGAAAGCATC	GTGGTCAAGG	AGGTTCCAAC	CCGAAATTTG
7351	AGAACATTGC	AGAAGGTTTA	AGAGCTCTCC	TGGCTAGGAG	TCACGTAGAA
7401	AGGACTACCG	ACGAAGGAAC	TTGGGTCGCC	GGTGTGTTCG	TATATGGAGG
7451	TAGTAAGACC	TCCCTTTACA	ACCTAAGGCG	AGGAACTGCC	CTTGCTATTC

G

FIG. 3h

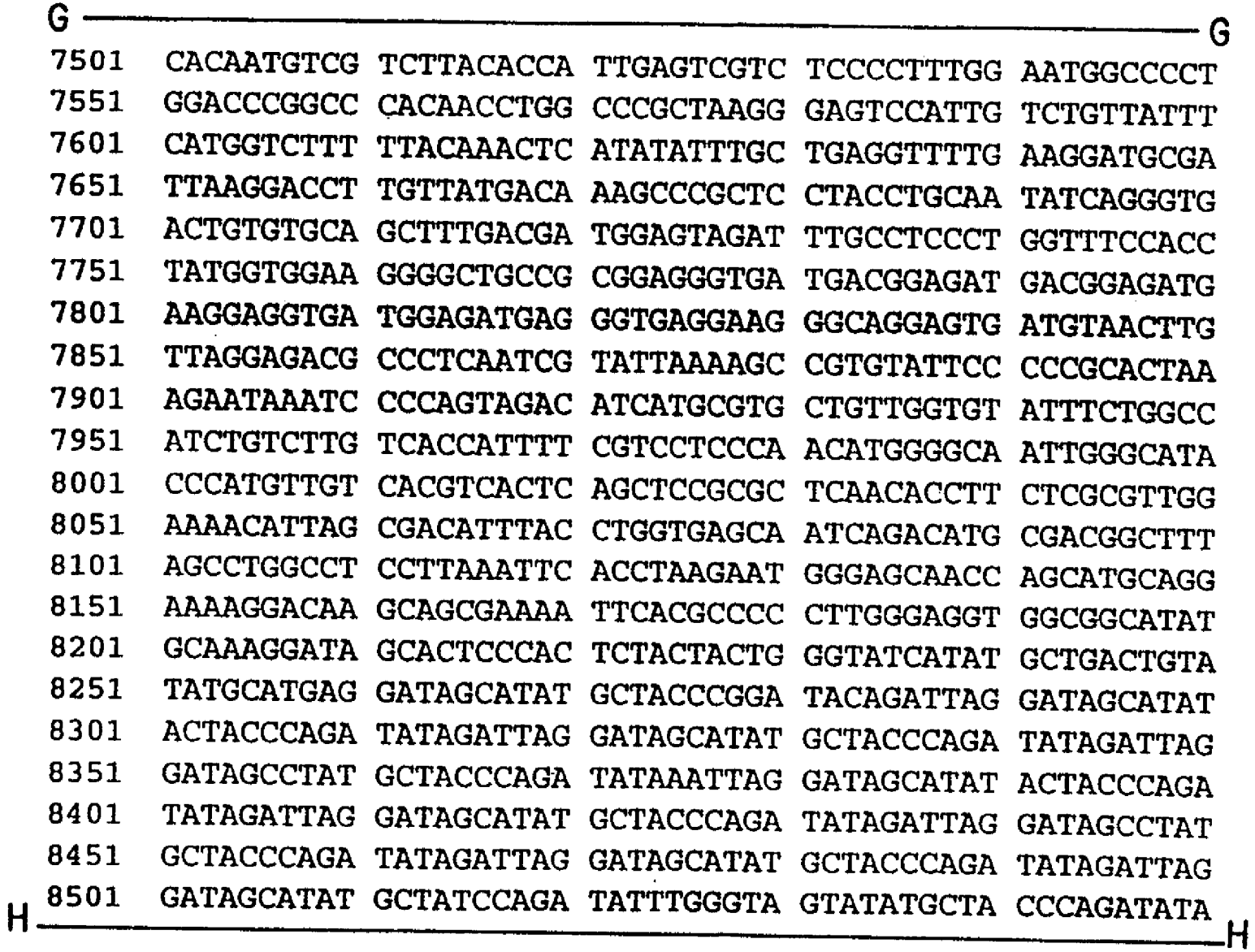


FIG. 3i

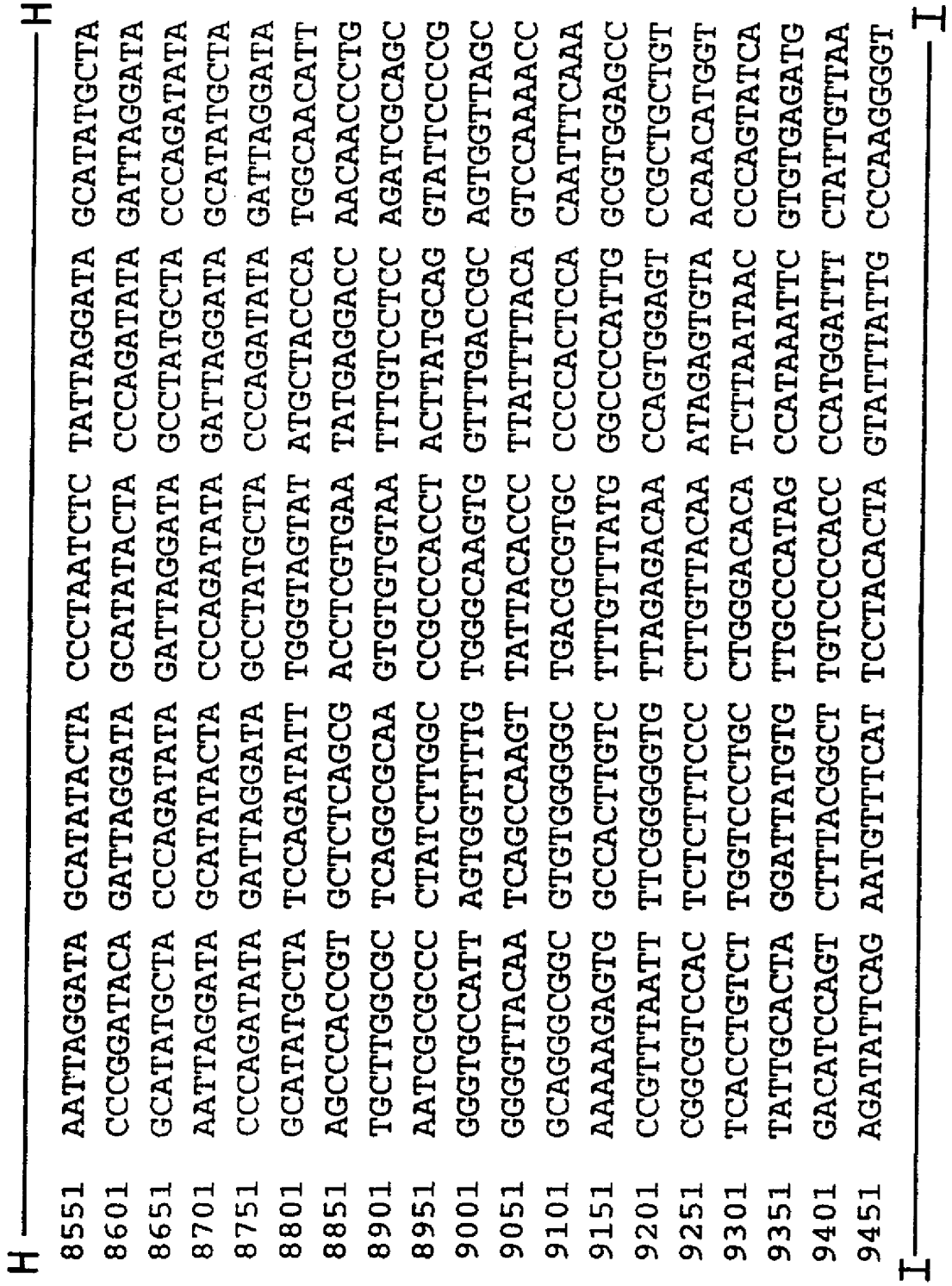


FIG. 3j

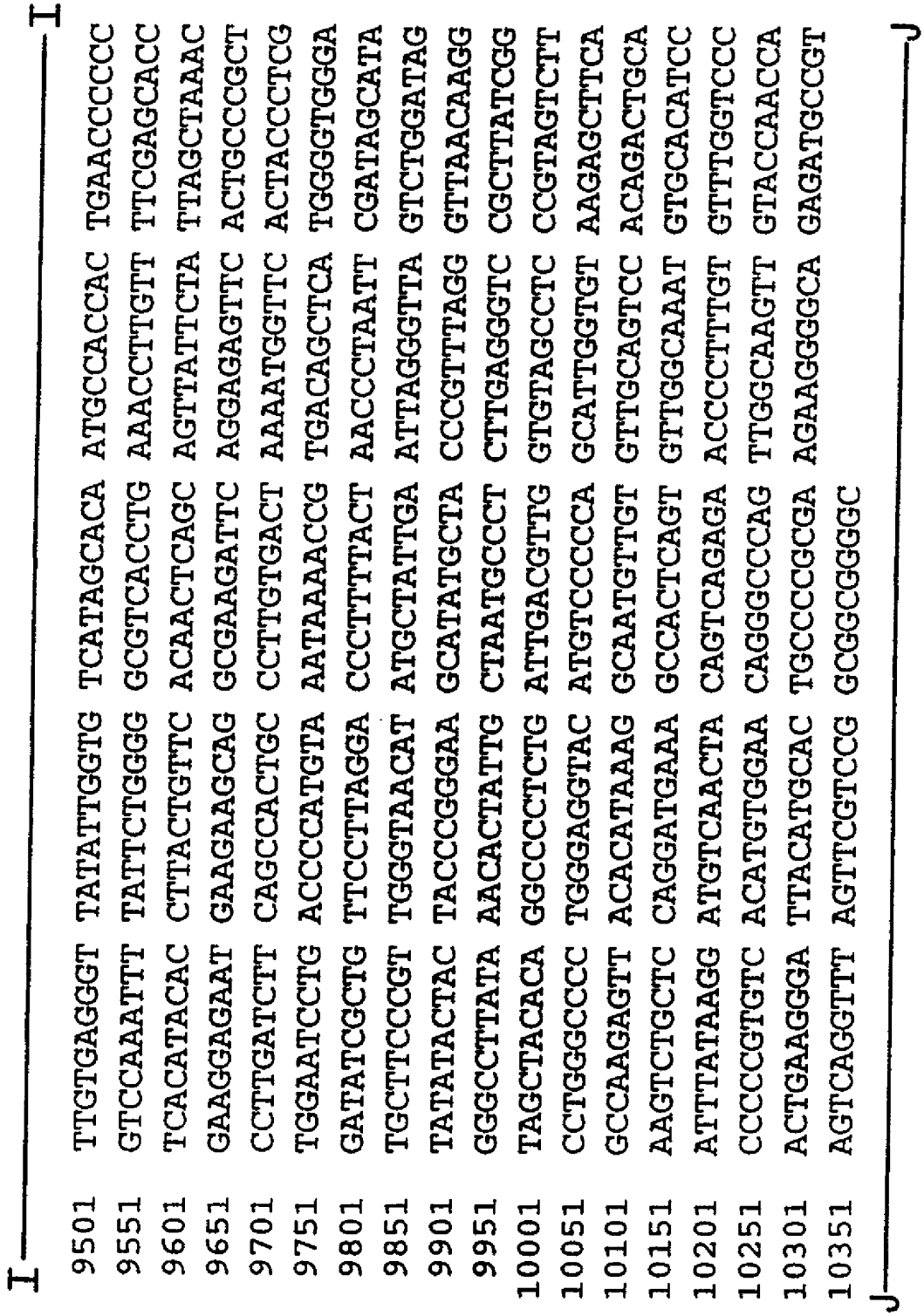

I
J
9501 TTGTGAGGGT TATATTGGTG TCATAGCACA ATGCCACCAC TGAACCCCCC
9551 GTCCAAATTT TATTCTGGGG GCGTCACCTG AAACCTTGTT TTCGAGCACC
9601 TCACATACAC CTTACTGTTC ACAACTCAGC AGTTATTCTA TTAGCTAAAC
9651 GAAGGAGAAT GAAGAAGCAG GCGAAGATTC AGGAGAGTTC ACTGCCCGCT
9701 CCTTGATCTT CAGCCACTGC CCTTGTGACT AAAATGGTTC ACTACCCTCG
9751 TGGAATCCTG ACCCCATGTA AATAAAACCG TGACAGCTCA TGGGGTGGGA
9801 GATATCGCTG TTCCTTAGGA CCCTTTTACT AACCCTAATT CGATAGCATA
9851 TGCTTCCCGT TGGGTAACAT ATGCTATTGA ATTAGGGTTA GTCTGGATAG
9901 TATATACTAC TACCCGGGAA GCATATGCTA CCCGTTTAGG GTTAACAAGG
9951 GGGCCTTATA AACACTATTG CTAATGCCCT CTTGAGGGTC CGCTTATCGG
10001 TAGCTACACA GGCCCCTCTG ATTGACGTTG GTGTAGCCTC CCGTAGTCTT
10051 CCTGGGCCCC TGGGAGGTAC ATGTCCCCCA GCATTGGTGT AAGAGCTTCA
10101 GCCAAGAGTT ACACATAAAG GCAATGTTGT GTTGCAGTCC ACAGACTGCA
10151 AAGTCTGCTC CAGGATGAAA GCCACTCAGT GTTGGCAAAT GTGCACATCC
10201 ATTTATAAGG ATGTCAACTA CAGTCAGAGA ACCCCTTTGT GTTTGGTCCC
10251 CCCCCGTGTC ACATGTGGAA CAGGGCCCAG TTGGCAAGTT GTACCAACCA
10301 ACTGAAGGGA TTACATGCAC TGCCCCGCGA AGAAGGGGCA GAGATGCCGT
10351 AGTCAGGTTT AGTTCGTCCG GCGGCGGGGC

FIG. 4a

```
   1  GGCGTAATCT GCTGCTTGCA AACAAAAAAA CCACCGCTAC CAGCGGTGGT
  51  TTGTTTGCCG GATCAAGAGC TACCAACTCT TTTTCCGAAG GTAACTGGCT
 101  TCAGCAGAGC GCAGATACCA AATACTGTCC TTCTAGTGTA GCCGTAGTTA
 151  GGCCACCACT TCAAGAACTC TGTAGCACCG CCTACATACC TCGCTCTGCT
 201  AATCCTGTTA CCAGTGGCTG CTGCCAGTGG CGATAAGTCG TGTCTTACCG
 251  GGTTGGACTC AAGACGATAG TTACCGGATA AGGCGCAGCG GTCGGGCTGA
 301  ACGGGGGGTT CGTGCACAGA GCCCAGCTTG GAGCGAACGA CCTACACCGA
 351  ACTGAGATAC CTACAGCGTG AGCATTGAGA AAGCGCCACG CTTCCCGAAG
 401  GGAGAAAGGC GGACAGGTAT CCGGTAAGCG GCAGGGTCGG AACAGGAGAG
 451  CGCACGAGGG AGCTTCCAGG GGGAAACGCC TGCTATCTTT ATGATCCTGT
 501  CGGGTTTCGC CACCTCTGAC TTGAGCGTCG ATTTTTGTGA TGCTCGTCAG
 551  GGGGGCGGAG CCTATGGAAA AACGCCAGCA ACGCAAGCTA GCTTCTAGCT
 601  AGAAATTGTA AACGTTAATA TTTTGTTAAA ATTCGCGTTA AATTTTTGTT
 651  AAATCAGCTC ATTTTTTAAC CAATAGGCCG AAATCGGCAA AATCCCTTAT
 701  AAATCAAAAG AATAGCCCGA GATAGGGTTG AGTGTTGTTC CAGTTTGGAA
 751  CAAGAGTCCA CTATTAAAGA ACGTGGACTC CAACGTCAAA GGGCGAAAAA
 801  CCGTCTATCA GGGCGATGGC CGCCCACTAC GTGAACCATC ACCCAAATCA
 851  AGTTTTTTGG GGTCGAGGTG CCGTAAAGCA CTAAATCGGA ACCCTAAAGG
 901  GAGCCCCCGA TTTAGAGCTT GACGGGGAAA GCCGGCGAAC GTGGCGAGAA
 951  AGGAAGGGAA GAAAGCGAAA GGAGCGGGCG CTAGGGCGCT GGCAAGTGTA
1001  GCGGTCACGC TGCGCGTAAC CACCACACCC GCCGCGCTTA ATGCGCCGCT
1051  ACAGGGCGCG TACTATGGTT GCTTTGACGA GACCGTATAA CGTGCTTTCC
```

A —— A

FIG. 4b

A — A

1101	TCGTTGGAAT	CAGAGCGGGA	GCTAAACAGG	AGGCCGATTA	AAGGGATTTT
1151	AGACAGGAAC	GGTACGCCAG	CTGGATTACC	GCGGTCTTTC	TCAACGTAAC
1201	ACTTTACAGC	GGCGCGTCAT	TTGATATGAT	GCGCCCCGCT	TCCCGATAAG
1251	GGAGCAGGCC	AGTAAAAGCA	TTACCCGTGG	TGGGGTTCCC	GAGCGGCCAA
1301	AGGGAGCAGA	CTCTAAATCT	GCCGTCATCG	ACTTCGAAGG	TTCGAATCCT
1351	TCCCCCACCA	CCATCACTTT	CAAAAGTCCG	AAAGAATCTG	CTCCCTGCTT
1401	GTGTGTTGGA	GGTCGCTGAG	TAGTGCGCGA	GTAAAATTTA	AGCTACAACA
1451	AGGCAAGGCT	TGACCGACAA	TTGCATGAAG	AATCTGCTTA	GGGTTAGGCG
1501	TTTTGCGCTG	CTTCGCGATG	TACGGGCCAG	ATATACGCGT	TGACATTGAT
1551	TATTGACTAG	TTATTAATAG	TAATCAATTA	CGGGGTCATT	AGTTCATAGC
1601	CCATATATGG	AGTTCCGCGT	TACATAACTT	ACGGTAAATG	GCCCGCCTGG
1651	CTGACCGCCC	AACGACCCCC	GCCCATTGAC	GTCAATAATG	ACGTATGTTC
1701	CCATAGTAAC	GCCAATAGGG	ACTTTCCATT	GACGTCAATG	GGTGGACTAT
1751	TTACGGTAAA	CTGCCCACTT	GGCAGTACAT	CAAGTGTATC	ATATGCCAAG
1801	TACGCCCCCT	ATTGACGTCA	ATGACGGTAA	ATGGCCCGCC	TGGCATTATG
1851	CCCAGTACAT	GACCTTATGG	GACTTTCCTA	CTTGGCAGTA	CATCTACGTA
1901	TTAGTCATCG	CTATTACCAT	GGTGATGCGG	TTTTGGCAGT	ACATCAATGG
1951	GCGTGGATAG	CGGTTTGACT	CACGGGGATT	TCCAAGTCTC	CACCCCATTG
2001	ACGTCAATGG	GAGTTTGTTT	TGGCACCAAA	ATCAACGGGA	CTTTCCAAAA
2051	TGTCGTAACA	ACTCCGCCCC	ATTGACGCAA	ATGGGCGGTA	GGCGTGTACG
2101	GTGGGAGGTC	TATATAAGCA	GAGCTCTCTG	GCTAACTAGA	GAACCCACTG

B — B

FIG. 4c

B

2151 CTTACTGGCT TATCGAAATT AATACGACTC ACTATAGGGA GACCCAAGCT B

2201 TCTAGAGATC CCTCGACCTC GAGATCCATT GTGCTGGCGC GGATTCTTTA

2251 TCACTGATAA GTTGGTGGAC ATATTATGTT TATCAGTGAT AAAGTGTCAA

2301 GCATGACAAA GTTGCAGCCG AATACAGTGA TCCGTGCCGG CCCTGGACTG

2351 TTGAACGAGG TCGGCGTAGA CGGTCTGACG ACACGCAAAC TGGCGGAACG

2401 GTTGGGGGTG CAGCAGCCGG CGCTTTACTG GCACTTCAGG AACAAGCGGG

2451 CGCTGCTCGA CGCACTGGCC GAAGCCATGC TGGCGGAGAA TCATACGCTT

2501 CGGTGCCGAG AGCCGACGAC GACTGGCGCT CATTTCTGAT CGGGAATCCC

2551 GCAGCTTCAG GCAGGCGCTG CTCGCCTACC GCCAGCACAA TGGATCTCGA

2601 GGGATCTTCC ATACCTACCA GTTCTGCGCC TGCAGGTCGC GGCCGCGACT

2651 CTAGAGGATC TTTGTGAAGG AACCTTACTT CTGTGGTGTG ACATAATTGG

2701 ACAAACTACC TACAGAGATT TAAAGCTCTA AGGTAAATAT AAAATTTTTA

2751 AGTGTATAAT GTGTTAAACT ACTGATTCTA ATTGTTGTGG TATTTTAGAT

2801 TCCAACCTAT GGAACTTATG AATGGGAGCA GTGGTGGAAT GCCTTTAATG

2851 AGGAAAACCT GTTTTGCTCA GAAGAAATGC CATCTAGTGA TGATGAGGCT

2901 ACTGCTGACT CTCAACATTC TACTCCTCCA AAAAAGAAGA GAAAGGTAGA

2951 AGACCCCAAG GACTTTCCTT CAGAATTGGT AAGTTTTTTG AGTCATGCTG

3001 TGTTTAGTAA TAGAACTCTT GCTTGCTTTG CTATTTACAC CACAAAGGAA

3051 AAAGCTGCAC TGCTATACAA GAAAATTATG GAAAAATATT TGATGTATAG

3101 TGCCTTGACT AGAGATCATA ATCAGCCATA CCACATTTGT AGAGGTTTTA

3151 CTTGCTTTAA AAAACCTCCC ACACCTCCCC CTGAACCTGA AACATAAAAT

3201 GAATGCAATT GTTGTTGTTA ACTTGTTTAT TGCAGCTTAT AATGGTTACA C

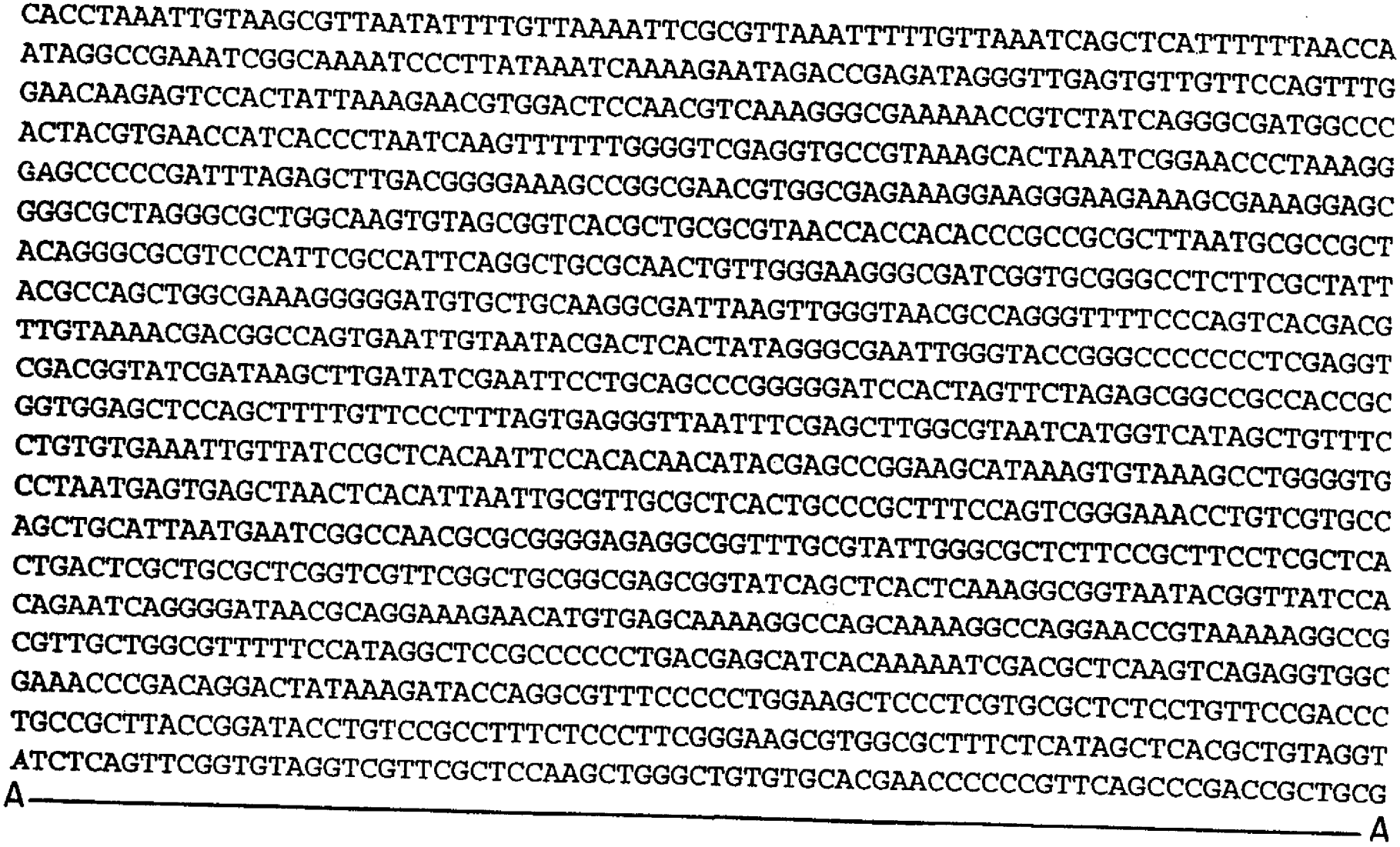
FIG. 4d

C

```
3251  AATAAAGCAA TAGCATCACA AATTTCACAA ATAAAGCATT TTTATCACTG
3301  CATTCTAGTT GTGGTTTGTC CAAACTCATC AATGTATCTT ATCATGTCTG
3351  GATCCCGCCA TGGTATCAAC GCCATATTTC TATTTACAGT AGGGACCTCT
3401  TCGTTGTGTA GGTACCGCTG TATTCCTAGG GAAATAGTAG AGGCACCTTG
3451  AACTGTCTGC ATCAGCCATA TAGCCCCCGC TGTTCGACTT ACAAACACAG
3501  GCACAGTACT GACAAACCCA TACACCTCCT CTGAAATACC CATAGTTGCT
3551  AGGGCTGTCT CCGAACTCAT TACACCCTAC CAAGTGAGAG CTGTAATTTC
3601  GCGATCAAGG GCAGCGAGGG CTTCTCCAGA TAAAATAGCT TCTGCCGAGA
3651  GTCCCGTAAG GGTAGACACT TCAGCTAATC CCTCGATGAG GTCTACTAGA
3701  ATAGTCAGTG CGGCTCCCAT TTTGAAAATT CACTTACTTG ATCAGCTTCA
3751  GAAGATGGGC GAGGGCCTCC AACACAGTAA TTTTCCTCCC GACTCTTAAA
3801  ATAGAAAATG TCAAGTCAGT TAAGGAGGAA GTGGACTAAC TGACGCAGCT
3851  GGCCGTGCGA CATCCTCTTT TAATTAGTTG CTAGGCAACG CCCTCCAGAG
3901  GGCGTGTGGT TTTGCAAGAG GAAGCAAAAG CCTCTCCACC CAGGCCTAGA
3951  ATGTTTCCAC CCAATCATTA CTATGACAAC AGCTGTTTTT TTTAGTATTA
4001  AGCAGAGGCC GGGGACCCCT GGGCCCGCTT ACTCTGGAGA AAAAGAAGAG
4051  AGGCATTGTA GAGGCTTCCA GAGGCAACTT GTCAAAACAG GACTGCTTCT
4101  ATTTCTGTCA CACTGTCTGG CCCTGTCACA AGGTCCAGCA CCTCCATACC
4151  CCCTTTAATA AGCAGTTTGG GAACGGGTGC GGGTCTTACT CCGCCCATCC
4201  CGCCCCTAAC TCCGCCCAGT TCCGCCCATT CTCCGCCCCA TGGCTGACTA
4251  ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCCTCGGCCT CTGAGCTATT
4301  CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG CCTAGGCTTT TGCAAAAAGC
4351  TAATTC
```

C

FIG. 5a



SUBSTITUTE SHEET (RULE 26)

FIG. 5b

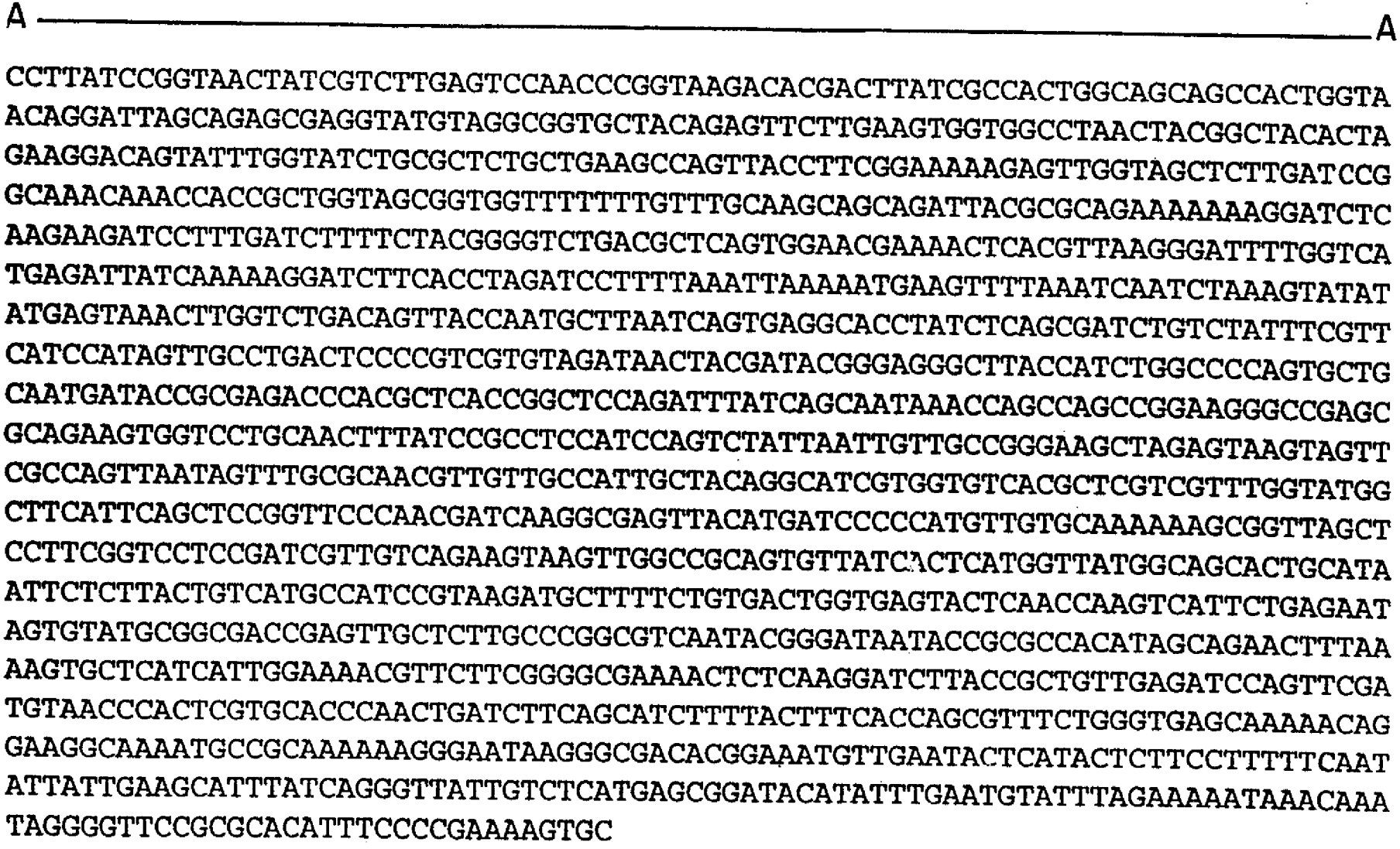


FIG. 6a

```
   1 CTGTGGAATG TGTGTCAGTT AGGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT
  61 ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCAGGTGTG GAAAGTCCCC AGGCTCCCCA
 121 GCAGGCAGAA GTATGCAAAG CATGCATCTC AATTAGTCAG CAACCATAGT CCCGCCCCTA
 181 ACTCCGCCCA TCCCGCCCCT AACTCCGCCC AGTTCCGCCC ATTCTCCGCC CCATGGCTGA
 241 CTAATTTTTT TTATTTATGC AGAGGCCGAG GCCGCCTCGG CCTCTGAGCT ATTCCAGAAG
 301 TAGTGAGGAG GCTTTTTTGG AGGCCTAGGC TTTTGCAAAA AGCTTGCATG CCTGCAGGTC
 361 GGCCGCCACG ACCGGTGCCG CCACCATCCC CTGACCCACG CCCCTGACCC CTCACAAGGA
 421 GACGACCTTC CATGACCGAG TACAAGCCCA CGGTGCGCCT CGCCACCCGC GACGACGTCC
 481 CCCGGGCCGT ACGCACCCTC GCCGCCGCGT TCGCCGACTA CCCCGCCACG CGCCACACCG
 541 TCGACCCGGA CCGCCACATC GAGCGGGTCA CCGAGCTGCA AGAACTCTTC CTCACGCGCG
 601 TCGGGCTCGA CATCGGCAAG GTGTGGGTCG CGGACGACGG CGCCGCGGTG GCGGTCTGGA
 661 CCACGCCGGA GAGCGTCGAA GCGGGGGCGG TGTTCGCCGA GATCGGCCCG CGCATGGCCG
 721 AGTTGAGCGG TTCCCGGCTG GCCGCGCAGC AACAGATGGA AGGCCTCCTG GCGCCGCACC
 781 GGCCCAAGGA GCCCGCGTGG TTCCTGGCCA CCGTCGGCGT CTCGCCCGAC CACCAGGGCA
 841 AGGGTCTGGG CAGCGCCGTC GTGCTCCCCG GAGTGGAGGC GGCCGAGCGC GCCGGGGTGC
 901 CCGCCTTCCT GGAGACCTCC GCGCCCCGCA ACCTCCCCTT CTACGAGCGG CTCGGCTTCA
 961 CCGTCACCGC CGACGTCGAG GTGCCCGAAG GACCGCGCAC CTGGTGCATG ACCCGCAAGC
1021 CCGGTGCCTG ACGCCCGCCC CACGACCCGC AGCGCCCGAC CGAAAGGAGC GCACGACCCC
1081 ATGGCTCCGA CCGAAGCCGA CCCGGGCGGC CCCGCCGACC CCGCACCCGC CCCCGAGGCC
1141 CACCGACTCT AGAGGATCAT AATCAGCCAT ACCACATTTG TAGAGGTTTT ACTTGCTTTA
1201 AAAAACCTCC CACACCTCCC CCTGAACCTG AAACATAAAA TGAATGCAAT TGTTGTTGTT
```

A——A

FIG. 6b

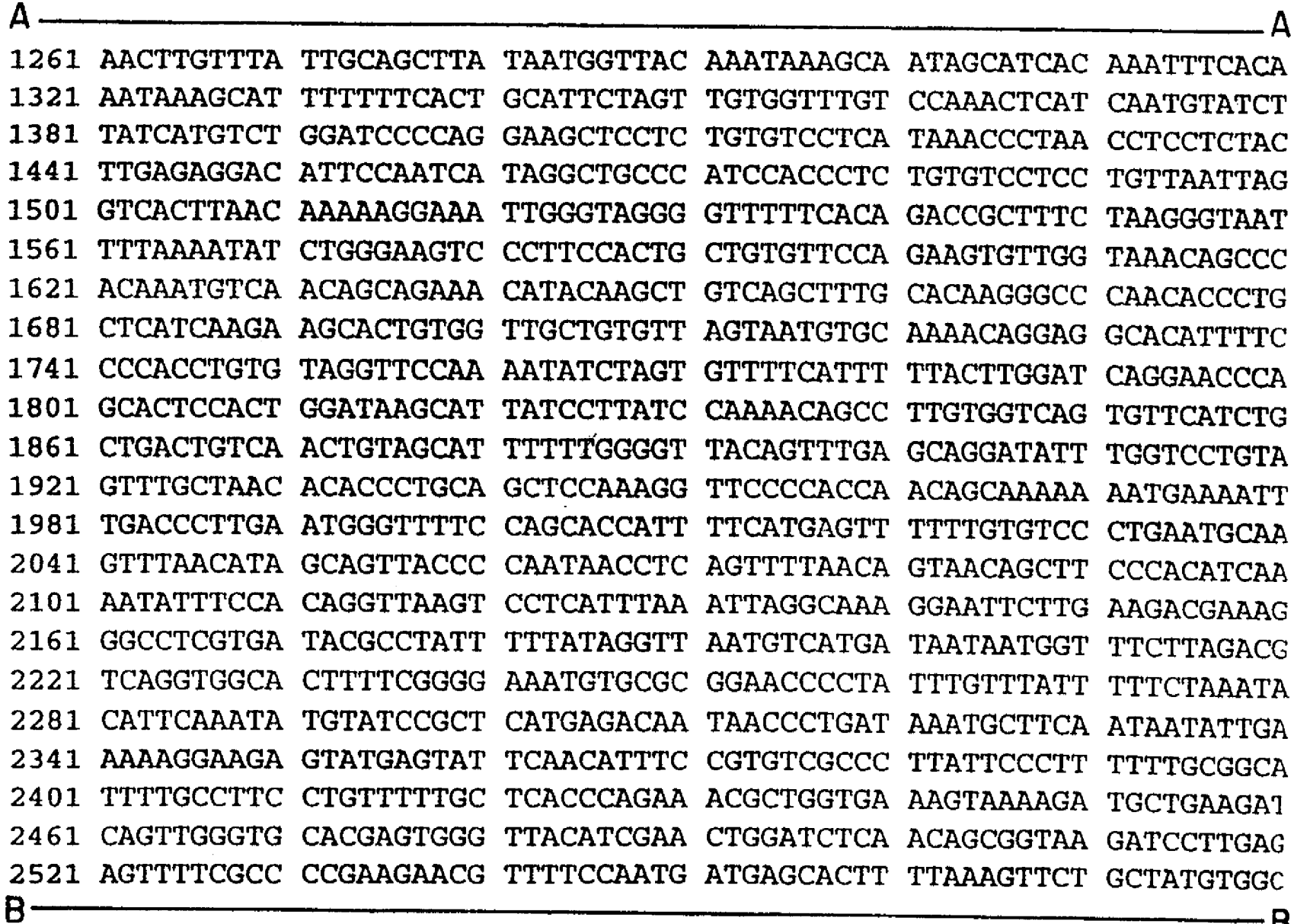


FIG. 6c

B

```
2581 GCGGTATTAT CCCGTGTTGA CGCCGGGCAA GAGCAACTCG GTCGCCGCAT ACACTATTCT
2641 CAGAATGACT TGGTTGAGTA CTCACCAGTC ACAGAAAAGC ATCTTACGGA TGGCATGACA
2701 GTAAGAGAAT TATGCAGTGC TGCCATAACC ATGAGTGATA ACACTGCGGC CAACTTACTT
2761 CTGACAACGA TCGGAGGACC GAAGGAGCTA ACCGCTTTTT TGCACAACAT GGGGGATCAT
2821 GTAACTCGCC TTGATCGTTG GGAACCGGAG CTGAATGAAG CCATACCAAA CGACGAGCGT
2881 GACACCACGA TGCCTGCAGC AATGGCAACA ACGTTGCGCA AACTATTAAC TGGCGAACTA
2941 CTTACTCTAG CTTCCCGGCA ACAATTAATA GACTGGATGG AGGCGGATAA AGTTGCAGGA
3001 CCACTTCTGC GCTCGGCCCT TCCGGCTGGC TGGTTTATTG CTGATAAATC TGGAGCCGGT
3061 GAGCGTGGGT CTCGCGGTAT CATTGCAGCA CTGGGGCCAG ATGGTAAGCC CTCCCGTATC
3121 GTAGTTATCT ACACGACGGG GAGTCAGGCA ACTATGGATG AACGAAATAG ACAGATCGCT
3181 GAGATAGGTG CCTCACTGAT TAAGCATTGG TAACTGTCAG ACCAAGTTTA CTCATATATA
3241 CTTTAGATTG ATTTAAAACT TCATTTTTAA TTTAAAAGGA TCTAGGTGAA GATCCTTTTT
3301 GATAATCTCA TGACCAAAAT CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC
3361 GTAGAAAAGA TCAAAGGATC TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG
```

C

FIG. 6d

C ——————————————————————————————— C

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3421 CAAACAAAAA AACCACCGCT ACCAGCGGTG GTTTGTTTGC CGGATCAAGA GCTACCAACT
3481 CTTTTTCCGA AGGTAACTGG CTTCAGCAGA GCGCAGATAC CAAATACTGT CCTTCTAGTG
3541 TAGCCGTAGT TAGGCCACCA CTTCAAGAAC TCTGTAGCAC CGCCTACATA CCTCGCTCTG
3601 CTAATCCTGT TACCAGTGGC TGCTGCCAGT GGCGATAAGT CGTGTCTTAC CGGGTTGGAC
3661 TCAAGACGAT AGTTACCGGA TAAGGCGCAG CGGTCGGGCT GAACGGGGGG TTCGTGCACA
3721 CAGCCCAGCT TGGAGCGAAC GACCTACACC GAACTGAGAT ACCTACAGCG TGAGCTATGA
3781 GAAAGCGCCA CGCTTCCCGA AGGGAGAAAG GCGGACAGGT ATCCGGTAAG CGGCAGGGTC
3841 GGAACAGGAG AGCGCACGAG GGAGCTTCCA GGGGGAAACG CCTGGTATCT TTATAGTCCT
3901 GTCGGGTTTC GCCACCTCTG ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG
3961 AGCCTATGGA AAAACGCCAG CAACGCGGCC TTTTTACGGT TCCTGGCCTT TTGCTGGCCT
4021 TTTGCTCACA TGTTCTTTCC TGCGTTATCC CCTGATTCTG TGGATAACCG TATTACCGCC
4081 TTTGAGTGAG CTGATACCGC TCGCCGCAGC CGAACGACCG AGCGCAGCGA GTCAGTGAGC
4141 GAGGAAGCGG AAGAGCGCCT GATGCGGTAT TTTCTCCTTA CGCATCTGTG CGGTATTTCA
4201 CACCGCATAT GGTGCACTCT CAGTACAATC TGCTCTGATG CCGCATAGTT AAGCCAG
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FIG. 7

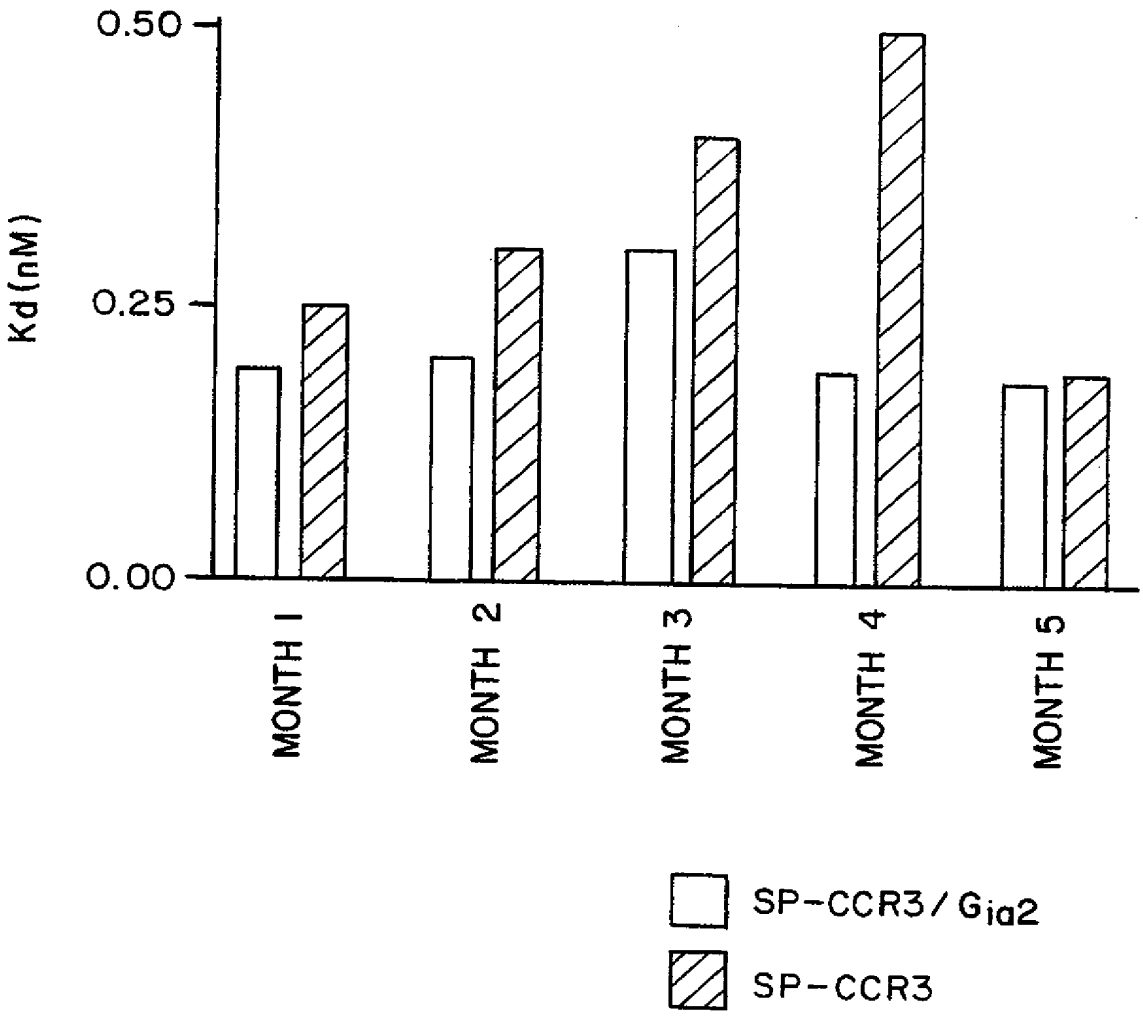
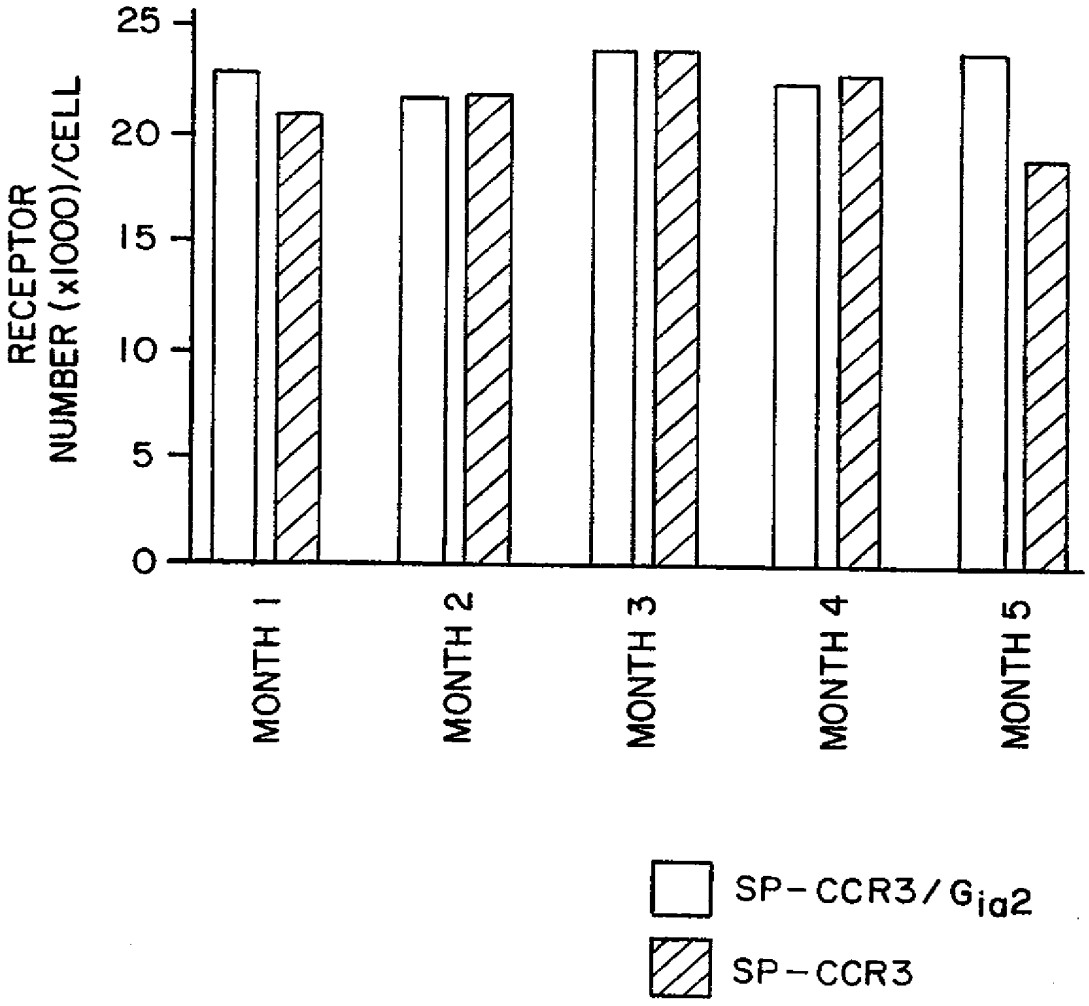


FIG. 8


RECEPTOR NUMBER (x1000)/CELL
25
20
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MONTH 1
MONTH 2
MONTH 3
MONTH 4
MONTH 5
SP-CCR3/Gia2
SP-CCR3

FIG. 9

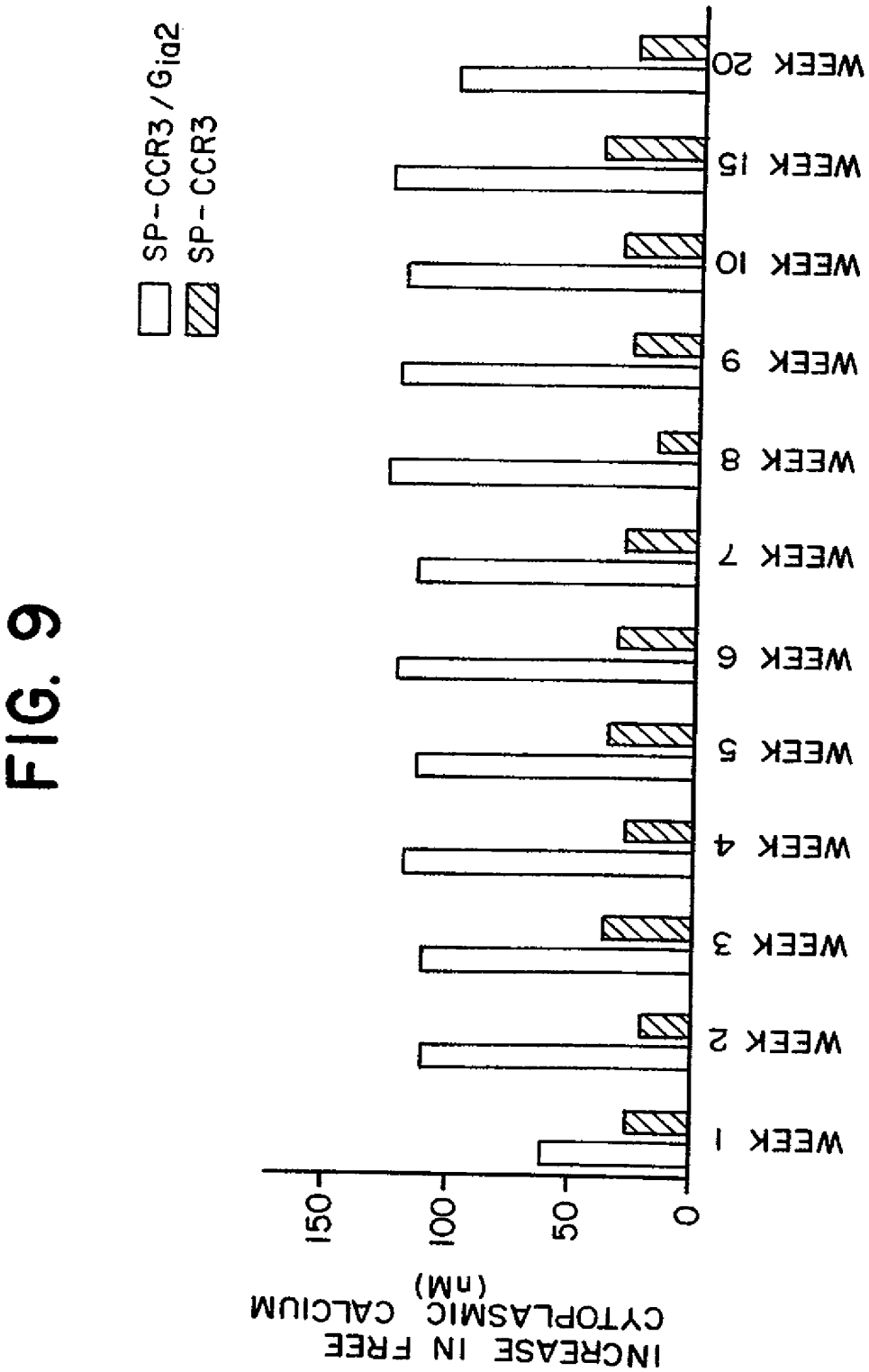


FIG. 10

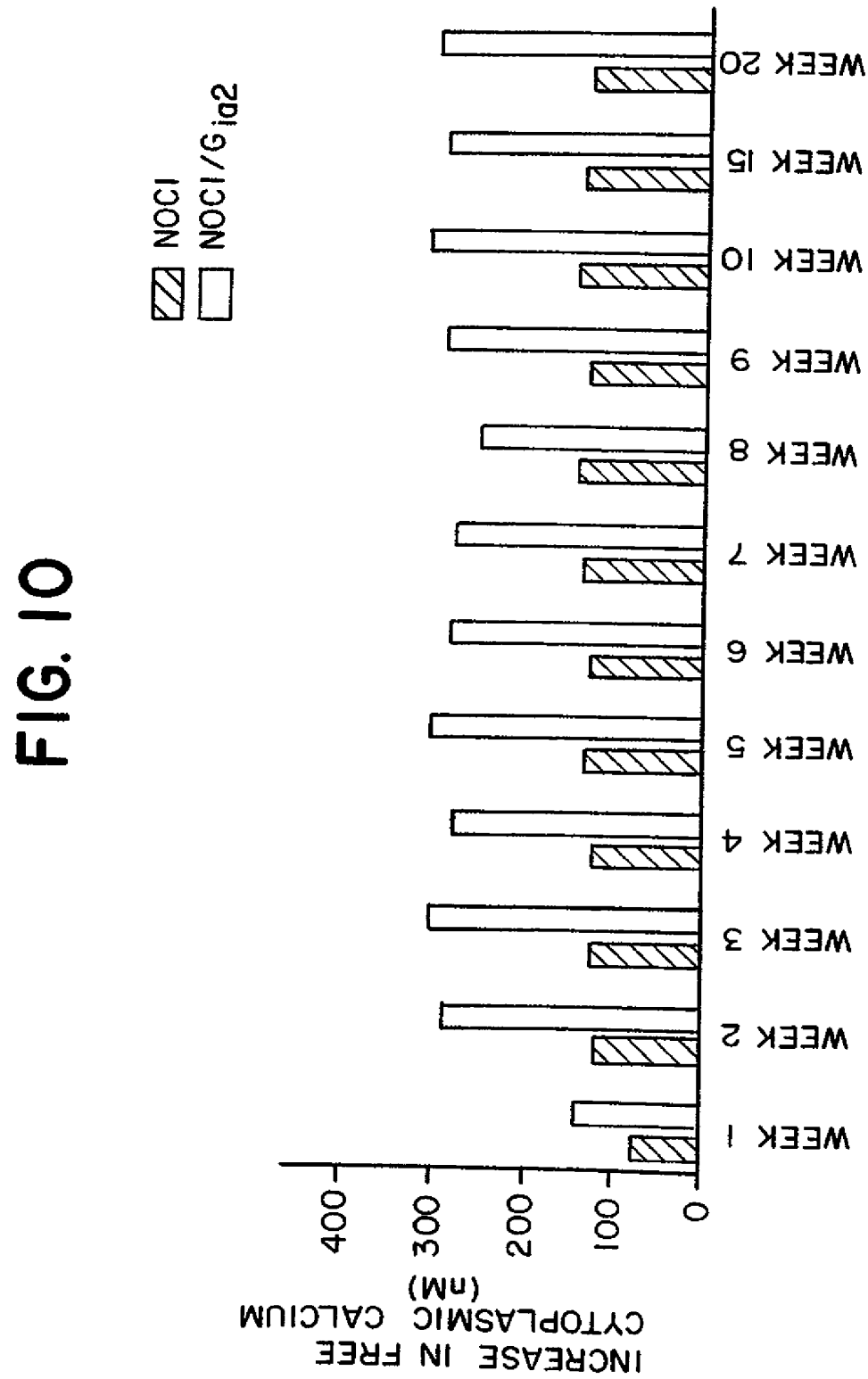


FIG. 11

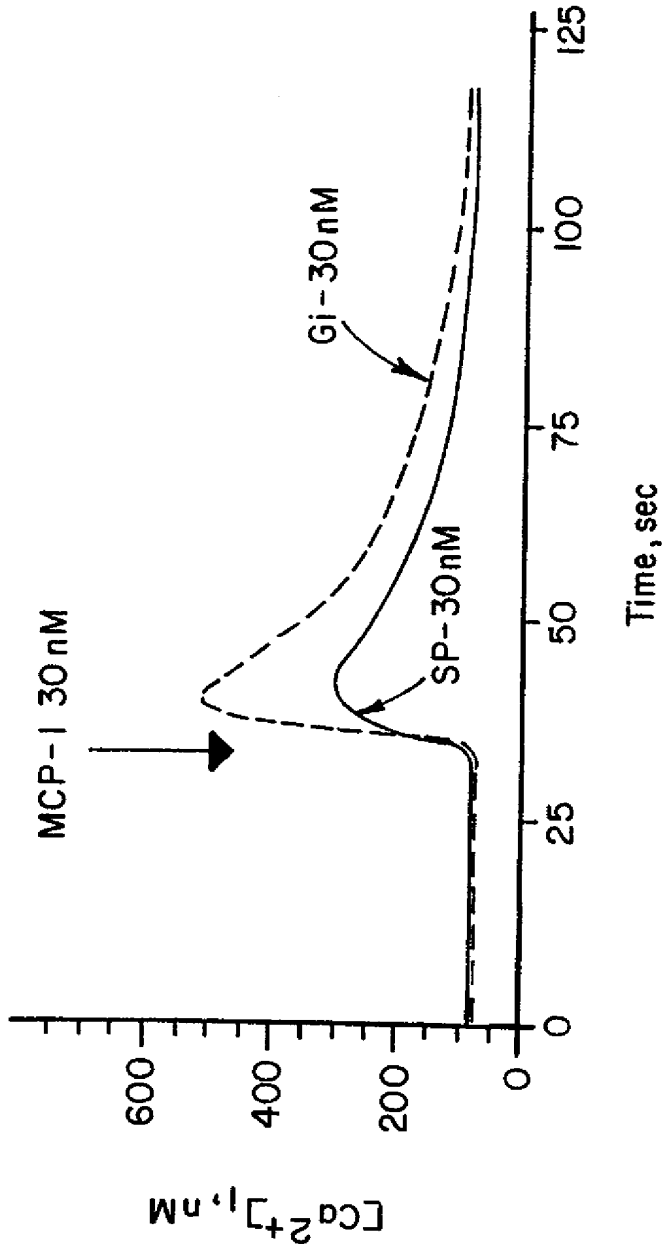
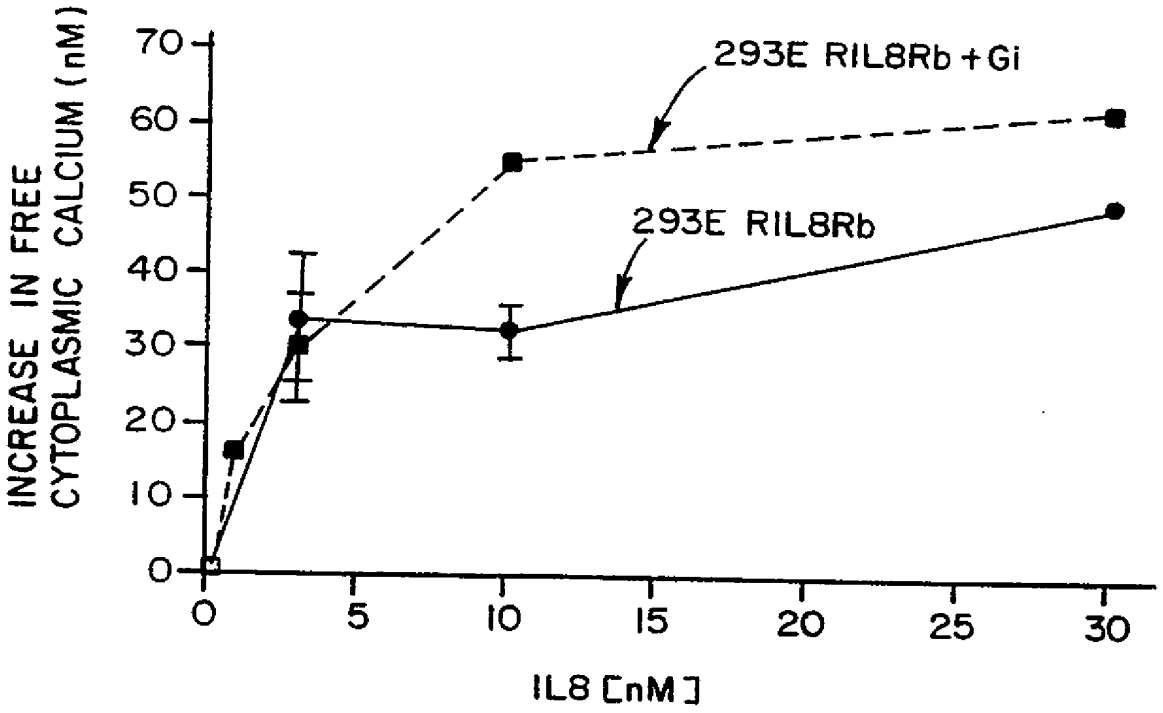


FIG. 12



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02852

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/50, 33/52; C07K 14/705; C12N 15/00, 15/12
US CL : 435/7.2, 69.1, 320.1; 536/23.1; 530/402
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 69.1, 320.1; 536/23.1; 530/402

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KUANG, Y. et al. Selective G Protein Coupling by C-C Chemokine Receptors. J. Biol. Chem. 23 February 1996, Vol. 271, No. 8, pages 3975-3978, see entire document.	1-35
Y	HERRLICH, A et al. Involvment of Gs and Gi Proteins in Dual Coupling of the Luteninizing Hormone Receptor to Adenylyl Cyclase and Phospholipase C. J. Biol. Chem. 12 July 1996, Vol. 271, No. 28, pages 16764-16772, see entire document.	1-35
Y	DAMAJ, B. B. et al. Physical Association of Gi2 Alpha with Interleukin-8 Receptors, J. Biol. Chem. 31 May 1996, Vol. 271, No. 22, pages 12783-12789, see entire document.	1-35

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
28 MAY 1999	12 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer D. Lawrence for NIRMAL S. BASI Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02852

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BERVEN, L. A. et al. Evidence That the Pertussis Toxin-sensitive Trimeric GTP-binding Protein Gi2 is required for Agonist- and Store-Activated Ca2+ Inflow in Hepatocytes. J. Biol. Chem. 27 October 1995, Vol. 270, No. 43, pages 25893-25897, see entire document.	1-35
Y	MAGOVCEVIC, I. et al. Regional Localization of the Human G Protein Alpha i2 (GNA12) Gene: Assignment to 3p21 and a Related Sequence (GNA12L) to 12p12-p13. Genomics, 1992, Vol. 12, pages 125-129, see entire document.	1-35
Y	MILLER, B. A. et al. Role of Pertussis Toxin-Sensitive Guanosine Triphosphate-Binding Proteins in the Response of Erythroblasts to Erythropoietin. Blood. 01 February 1991, Vol. 77, No. 3, pages 486-492, see entire document.	1-35
Y	MILLER, B. A. et al. G-protein Alpha Subunit Gi-alpha 2 Mediates Erythopoietin Signal transduction in Human Erythroid Precursors. J. Clin. Invest. 1996, Vol. 98, No. 8, pages 1728-1736, see entire document.	1-35
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Y	ZHU, x. et al. G Protein subunits and the Stimulation of Phospholipase C by Gs- and Gi-coupled Receptors: Lack of Receptor Selectivity of G-alpha-16 and Evidence for a Synergic Interaction between G-beta-gamma and Alpha-subunit of a Receptor-Activated G Protein. Proc. Natl. Acad. Sci. USA, April 1996, Vol. 93, pages 2827-2831, see entire document.	1-35
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Y	DORN, G. W. et al. Alpha21-Adrenergic Receptor Stimulated calcium Release is Transduced by Gi-Associated G-beta-gamma-Mediated Activation of Phospholipase C. Biochemistry, 1997, Vol 36, pages 6415-6423, see entire document.	1-35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02852

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOYER, J. L. et al. Selective Activation of Phospholipase C by Recombinant G-Protein Alpha- and Beta-gamma-Subunits. J. Biol. Chem. 28 January 1994, Vol 269, No. 4, pages 2814-2819, see entire document.	1-35
Y	ARAGAY, A. M. et al. The G-alpha-q and G-alpha-11 Proteins Couple the Thyrotropin-releasing Hormone Receptor to Phospholipase C in GH3 Rat Pituitary Cells. J. Biol. Chem. 15 December 1992, Vol. 267, No. 35, pages 24983-24988, see entire document.	1-35
Y	BOYER, J. l. et al. Beta-Gamma-Subunit Activation of G-Protein-Reulated Phospholipase C. J. Biol. Chem. 15 December 1992, Vol. 267, No. 35, pages 25451-25456, see entire document.	1-35
Y	NEYLON, C. B. et al. Thrombin-induced Ca2+ Moblization in Vascular Smooth Muscle Utilizes a Slowly Ribosylating Pertussis Toxin-sensitive G protein. J. Biol. Chem. 15 April 1992, Vol. 267, No. 11, pages 7295-7302, see entire document.	1-35
Y	DUZIC, E. et al. Factors Determining the Specificity of Signal Transduction by Guanine Nucleotide-binding Protein-Coupled Receptors. J. Biol. Chem. 15 May 1992, Vol 267, No. 14, pages 9844-9851, see entire document.	1-35
Y	CLAPHAM, D. E. Intracellular Signalling: More Jobs for G-beta-gamma. Current Biology. 1996, Vol. 6, No. 7, pages 814-816, see entire document.	1-35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02852

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, JAPIO, BIOSIS, SCISEARCH, WPIDS
Search terms: g protein, calcium, gi, gia, gia2, gia3, gi1, gi2 gi3, chemokine, interleukin, fluorescence, fura, indo, fluo, phosphorylase C, episomal